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THE ANTIGENICITY OF THE VIRUS OF TRACHOMA *

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One of the more important clinical characteristics of trachoma is the tendency of the disease to recur in the same individual. While it is obviously difficult to determine whether repeated attacks of trachoma signify reinfection *de novo*, or instead an exacerbation of the virus remaining quiescent in the tissues during the arrested state, nevertheless the clinical indications are clear that immunity to trachoma must be ineffectual and, if present at all, certainly of low magnitude.

In pursuing studies already reported [1] on the etiology of trachoma the opportunity was presented to make experimental observations in both man and animal on the immunological responses invoked by the virus causing this disease. The observations resulting from this study appear to be of sufficiently broadened interest in the general field of viruses to merit their communication and thus place on record certain information hitherto unavailable.

METHODS

The suspension of virus consisted of tissues scraped from the conjunctiva of clinically active patients during the operation for grattage. The material from the eyes of each patient was suspended in 1.5 cc. of veal infusion broth and later used for testing, either alone or pooled with similar material from other patients.

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The tissues were ground in a sterile mortar without the use of abrasive, and their infectivity was determined by inoculating monkeys (*Macacus rhesus*) in one eye by swabbing the conjunctiva of the everted lid, and in the other eye by multiple pricking of the conjunctiva with a charged needle and then injecting subconjunctivally 0.2 cc. or more of the suspension. Each test required material fresh from patients since the virus becomes inactivated rapidly.

Neutralization tests were performed in general by mixing equal quantities of undiluted or diluted serum and material obtained from patients in the manner described. The mixtures were then incubated in a water bath at 37° C. for 30 to 60 minutes, as will be brought out later, and inoculated in monkeys as indicated above.

Antiserums were obtained by injecting intravenously the triturated suspensions of grattage material. Since immunization was dependent on the availability of fresh active tissues rather than on some prearranged schedule, it was fortunate that injections were actually given every 3 to 5 days over a period of 2 months. It was not possible, however, to ascertain the infectivity of the tissues used for immunization because the incubation period of the disease exceeds the period of survivability of the virus.[2] It was felt, nevertheless, that careful selection of patients, prolonged immunization, and the established fact[3] that half the patients taken at random are in the infectious stage, would compensate for this omission. The majority of the tissues injected contained inclusion bodies, so that if, as seems probable, these structures are expressions or phases of the virus itself, they should add correspondingly to the process of immunization. Animals were bled by puncturing the heart 10 days following the final injection, and the serums were subsequently used for the tests to be recorded.

Experimental Observations

The determination of possibly acquired immunity in monkeys following recovery from artificially induced infection has already been referred to in a previous communication.[3] In order that the immunological reactions associated with trachoma may be brought together in final and complete form, however, it is desirable to include in this report additional examples bearing on this question. As soon after recovery as active material was available,

monkeys were subjected to inoculation as described above. In order to illustrate the reactions observed, the data from 5 typical animals are given in Table I. It will be seen that in each case infection ensued in somewhat the same manner as observed in the previous instance and ran a similar course. The interval between infection and reinoculation varied from 2 weeks to 3 months, so that no particular emphasis can be placed on the differences in time between recovery and subsequent inoculation as a factor in the development of the later infection. The evidence for continued susceptibility in recovered monkeys can be amplified by numerous examples in this laboratory. In fact, it is because of the frequency of this observation that it is now our custom to reserve

TABLE I

Effect of Recovery from Experimental Trachoma on Later Infection

Animal number	Synopsis, first infection		Interval after recovery	Synopsis, later infection	
	Incubation period	Duration		Incubation period	Duration
	days			*days*	
4	14	16 months	3 months	10	6 months (died)
2–4	9	6 weeks	2½ months	7	5 weeks
3–5	6	10 weeks	2½ months	18	12 weeks
8	21	11 weeks	2 weeks	6	7 weeks
9	10	10 weeks	3 weeks	17	9 weeks

recovered animals for future experiments, since having been infected successfully in the past they are of proved susceptibility to trachoma and thus automatically exclude in the analysis of data the possibility of individual resistance as responsible for the absence of infection following inoculation of trachomatous tissues.

Subsequent experiments were undertaken to determine, if possible, the occurrence of antibodies in the blood of patients suffering with trachoma. Blood was taken from a number of individuals who had been afflicted with the disease over periods varying from a few months to several years. In some experiments defibrinated blood was used and in others only serum. Mixtures of grattage material and blood, the ultimate concentration of which varied from 1:1 to 1:5, were incubated in a water bath at 37° C. for 30 minutes and then introduced conjunctivally in monkeys. In no instance was it possible to show that the blood of patients neutralized active virus (*i.e.*, prevented infection when inoculated in

this form in susceptible monkeys). The resulting infection in the animals was as typical as that observed in monkeys inoculated with material alone, or material previously mixed and incubated with equivalent amounts of non-trachomatous serums. It is, therefore, fair to state that under the conditions outlined the blood of trachomatous patients contains no antibodies capable of neutralizing the specific virus.

Similar experiments were carried out with the serums of monkeys shortly after their recovery from experimental infection. The results in these instances confirmed the observations made with serums of patients.

It is, however, a well recognized fact among bacteriologists and immunologists interested in ophthalmological infections that infectious agents adapted to the conjunctiva very rarely stimulate formation of antibodies. Functionable antigens, such as those contained in pneumococcus, streptococcus, gonococcus, Koch-Weeks bacillus, and so on, are antigenically inert as far as antibodies in the general circulation are concerned unless, as is rarely the case, the organisms penetrate through the conjunctival barrier. Consequently, lack of measurable protective antibody in trachoma may imply as much an incompetency of the tissues involved to elaborate antibodies as an impotency on the part of the antigen to stimulate them. Thus, then, it became necessary to attempt artificial immunization of animals in order to define more accurately the essential conditions of the general immune response to the virus of trachoma.

Accordingly, 2 rabbits were immunized as described above, and because it may be possible that a virus will stimulate formation of antibodies only in animals susceptible to the infection it induces, monkeys were also studied. Two monkeys that had recovered from severe and prolonged experimental trachoma were used for the purpose. Both rabbits and monkeys received 16 injections of the human tissues as soon after collection from the patient as was possible, each injection consisting of 0.5 cc. or 1 cc. of the suspension. The serums subsequently obtained from these animals were then studied for the presence of neutralizing or protective antibodies.

Neutralization tests were conducted according to the technique described above. The tubes containing the mixtures of tissues

and serums were agitated from time to time during incubation and in each instance it was noticeable grossly that the serums both agglutinated and lysed the human cells. Smears made at the end of the incubation revealed a complete disappearance of red cells and less complete but still marked lysis of polymorphonuclear cells, with lymphocytes somewhat more resistant than the neutrophils. Perhaps as many as half of the epithelial cells from the conjunctiva were also lysed, while the others were in more or less normal condition. Since corollary experiments conducted in a similar manner with normal rabbit and monkey serums revealed no agglutinative or lytic effect on the human cells, this observation suggests at least that the conditions of the experiment were appropriate for the formation of antibodies in response to the active antigens present.

TABLE II

Protective Capacity of Rabbit and Monkey Antiserums in Experimental Trachoma

Experiment number	Antiserum		Period of fixation	Result of test
	Animal	Dilution		
			min.	
NT-3	Rabbit	1:1	30	No protection
	Monkey	1:1	30	No protection
NT-6	Rabbit	1:5	30	No protection
	Monkey	1:3	30	No protection
NT-7	Rabbit	1:1	60	No protection
	Monkey	1:1	60	No protection

Seven experiments were done in all, but in only three was the original material infectious for monkeys. The results of these three experiments are summarized in Table II. It will be seen that rabbit and monkey antiserums were used in parallel tests with the same human tissues. Serums were diluted 1:1, 1:3, and 1:5, and incubations were carried out for 30 and 60 minutes. Immediately on removal from the water-bath the mixtures were inoculated in monkeys, inducing infections typical in every way. Later, experiments were done in which guinea pig complement was added to the system, but also without influencing the infectivity of the virus in any measurable manner. It seems definite, therefore, that prolonged artificial immunization of rabbits or monkeys, the latter of verified susceptibility to trachoma, with human

tissues containing specific virus fails to stimulate antibodies capable of protecting monkeys against experimental trachomatous infection.

Discussion

The observations recorded in the present communication suggest that judged by increased resistance to experimental infection in monkeys the virus of trachoma is an ineffectual antigen. The lack of active immunity observed so commonly in patients has thus been duplicated experimentally in animals. In fact, because normal monkeys are frequently resistant to trachomatous infection, it has been found an accommodation to use over and over again recovered animals as of proved susceptibility. Records of individual monkeys, consequently, show that in this laboratory animals have been infected successively three, four, and even five times.

Since the serums from both patients and monkeys with trachoma contained no protective antibodies, experiments were undertaken to stimulate a general rather than a localized immunogenic reaction by injecting intravenously tissues from patients containing both active virus and inclusion bodies. While rabbits are resistant to infection and perhaps in consequence unsuited for immunization with the virus of trachoma, the monkeys studied, on the other hand, were of verified susceptibility, having recovered only recently from the experimental ocular infection. Yet their serums, just as those of the rabbits, were lacking in protective antibodies as determined by methods usually adopted for their detection. In the face of such evidence, therefore, it is difficult to escape the conclusion that the virus of trachoma is at best a poor antigen.

The data, however, cannot be interpreted to mean that the virus of trachoma is not an antigen. It is difficult to conceive of any active infectious agent as devoid of antigenicity; it is more probable that the antigen is of extremely low activity and the methods in use at the present time for the demonstration of antibodies are too crude to detect their low concentration. In any case, it must be admitted that whatever immune response is aroused by the virus of trachoma, it is not capable of combatting the active infection arising spontaneously in man or induced artificially in monkeys.

On considering the question of immunity from its obverse aspect, the host, it is obvious that the conjunctival and corneal epithelium (the only tissues involved in uncomplicated trachoma) participate only to a minor degree, if at all, in the elaboration of antibodies. Consequently, unless the infectious agent penetrates through the barrier set up by the conjunctiva, the conditions for active immunity and formation of antibody are only rarely established. In this particular instance the low virulence of the trachomatous virus creates a relatively mild reaction, thus precluding its invasion into the subconjunctival tissues and beyond; even when introduced repeatedly into the general circulation, moreover, it exerts antigenically an inadequate stimulation.

Summary and Conclusion

1. Clinical observation reveals very little if any immunity to trachoma.

2. It has not been possible to demonstrate increased resistance to experimental trachoma in monkeys following recovery from the infection.

3. The serum or defibrinated blood of patients with active infections of varying duration exerts no neutralizing or protective effect on the virus of trachoma.

4. The serum of infected or recovered monkeys contains no antibodies demonstrable by the usual methods of protection.

5. The serum of rabbits or susceptible monkeys receiving repeated intravenous injections of active trachomatous tissues contains similarly no antiviral substances.

6. It is concluded that the virus of trachoma is an impotent and ineffectual antigen.

REFERENCES

1. Julianelle, Louis A. The Etiology of Trachoma. The Commonwealth Fund Division of Publications, New York, 1938.

2. Julianelle, Louis A., and Harrison, R. Wendell. Studies on the infectivity of trachoma. VIII. Biology of the infectious agent. *Am. J. Ophth.*, 1938, **21**, 529–535.

3. Julianelle, Louis A., and Harrison, R. Wendell. Studies on the infectivity of trachoma. I. Transfer of a conjunctival infection to monkeys by means of trachomatous tissues. *Am. J. Ophth.*, 1934, **17**, 1035–1044.

THE REACTION TO KILLED TUBERCLE BACILLI IN NORMAL AND IMMUNIZED (SENSITIZED) RABBITS *

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The purpose of these experiments was to determine how the reaction to intraperitoneally injected killed tubercle bacilli is modified by previous skin injections of the same material. When living bacilli are injected into an experimental animal two parallel processes develop, namely, the growth of the injected organisms and the progressively changing response of the host. With the use of killed bacilli the first factor is eliminated. This simplification of the problem is especially desirable when the attempt is made to study the regressive phase of the cellular reaction. Approximately half of our rabbits received repeated intracutaneous or subcutaneous injections of killed tubercle bacilli before intraperitoneal injections were made, and the resulting sensitization was measured by tuberculin tests or by the reaction to killed tubercle bacilli or by both. The others received only intraperitoneal injections and served as controls.

EXPERIMENTAL PROCEDURE

Bovine tubercle bacilli of the virulent Ravenel strain grown on glycerin agar by Dr. Jules Freund were carefully removed from the surface of the culture and killed by heat at or above 100° C. The death of the bacilli was confirmed by injecting 5 mg. in suspension subcutaneously into guinea pigs. We used unbuffered normal saline as the diluent and the pH of the resulting suspensions was between 6.8 and 7.2 The suspensions were homogeneously opaque on gross examination, but small clumps of bacilli were found under the oil immersion lens.

Tuberculin tests were made by intracutaneous injection of 0.04 cc. of old tuberculin (human type) diluted to 0.2 cc. with 4 parts of saline.

The skin reactions after the intracutaneous injection of killed

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bacilli or of tuberculin were examined in 48 hours and designated as negative when the largest diameter of edema was less than 5 mm.; 1 plus, when it was from 5 to 9 mm.; 2 plus, from 10 to 19 mm.; 3 plus, from 20 to 29 mm.; 4 plus, from 30 to 39 mm.; and 5 plus, from 40 to 49 mm., in accordance with the procedure used in this laboratory.

Of 83 rabbits used in the experiments, 17 were eliminated by "snuffles," pneumonia or diarrhea before intraperitoneal injection was performed. Two others died after peritoneal injection, 1 from pneumonia and the other from diarrhea. Two animals with extensive subcutaneous caseation but no peritoneal lesion were not included because it was evident that the inoculating needle had not entered the peritoneal cavity. The remaining 62 rabbits form the basis of this report. In most of the animals 5 cc. of saline was injected into the peritoneal cavity and the syringe containing the killed bacilli was then attached to the same needle. One cubic centimeter of a suspension containing 10 mg. of killed bacilli was injected and the lumen of the needle was rinsed into the peritoneum with 2 cc. of saline. In 3 animals (Nos. 9, 10 and 16) the suspension alone was injected. During the course of the experiment all of the animals except No. 31 increased in weight.

Designation of Extent of Peritoneal Lesions Found at Autopsy

The lesions found at autopsy were rated largely by the number and size of the tubercles in the peritoneum and omentum, but in rabbits that lived a week or less the redness of the omentum and the amount of intraperitoneal fluid were considered. The extent of tuberculosis is indicated as follows:

Tuberculosis was recorded as absent when no tubercles were found on gross or microscopic examination.

The lesion was recorded as doubtful when microscopic sections revealed groups of mononuclear cells, although gross examination revealed no tubercles or a very few indistinct fibrotic nodules on the peritoneum, with no change in the omentum.

One plus was used to indicate the presence of a few clearly defined tubercles with histological characteristics of tuberculosis in the peritoneum or the omentum and perhaps elsewhere.

Two plus was used when there were tubercles in moderate num-

ber, perhaps 10 small or 5 larger ones, on the peritoneum and omentum. The omentum in some instances contained a considerable amount of uninvolved tissue. In these animals and in those with more extensive lesions it was usual to find a caseous mass at the point where the needle perforated the peritoneum.

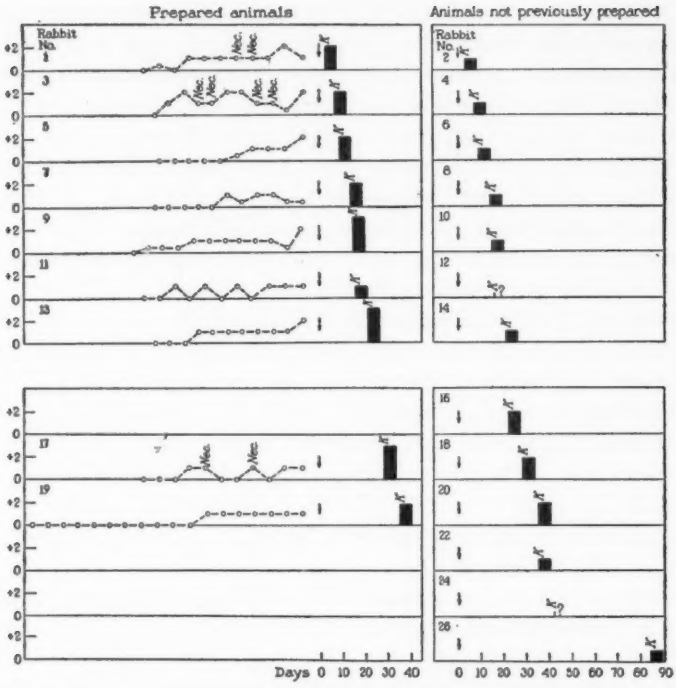
Three plus was used when there were many tubercles on the parietal and visceral peritoneum and the omentum, the latter being usually much thickened and uniformly matted. The number of tubercles under the dome of the diaphragm gave a measure of the extent of peritoneal involvement.

Four plus was used when the parietal peritoneum and the peritoneal surfaces of the liver, spleen, intestines and at least the greater part of the omentum were studded with tubercles. The peritoneal surface of the diaphragm was studded with discrete tubercles or was covered by a confluent mass of tuberculous tissue.

Experiment 1

Tubercle bacilli were autoclaved for 15 minutes at 15 pounds pressure, dried over phosphorus pentoxide and suspended in saline so that 2 mg. of dry bacilli (which were estimated to represent 10 mg. of moist bacilli) were present in each cubic centimeter. They were then placed in an Arnold steam sterilizer for 30 minutes at 98° C. All but 2 of the treated animals were given 10 to 12 intracutaneous injections each of 0.5 mg. of killed bacilli at approximately weekly intervals. Rabbit No. 19 received 18 injections of the same quantity and rabbit No. 9 received 6 injections of 0.2 mg., 5 of 0.5 mg., and 1 of 1 mg. The graphs in Text-Fig. 1 which give the extent of edema produced by these injections show the progress of sensitization. No tuberculin was used.

Sensitization in all of the animals of this experiment was scant and the diameter of the swelling produced by the intracutaneous injection of heat killed tubercle bacilli in no instance exceeded 10 to 19 mm. Nevertheless the extent of peritoneal tuberculosis produced by the injection of a large quantity of heat killed tubercle bacilli was uniformly greater in sensitized than in untreated animals. In this experiment sensitization was measured only with heat killed tubercle bacilli and in the absence of tuberculin tests it is not possible to determine the effect of the intraperitoneal injection or sensitization.



TEXT-FIG. 1. Lesions present in prepared rabbits of Experiment 1 and in rabbits not previously treated with killed tubercle bacilli. The broken lines show the extent of the edema produced by the intracutaneous injection of killed bacilli. "Nec." indicates that caseous necrosis was found at the site of intracutaneous injection in 7 days or less. The arrow indicates the day on which the intraperitoneal injection of 10 mg. of killed tubercle bacilli was made. The position of the black column indicates the duration of life, while its height is proportional to the extent of lesions at autopsy. "K" indicates the day animal was killed.

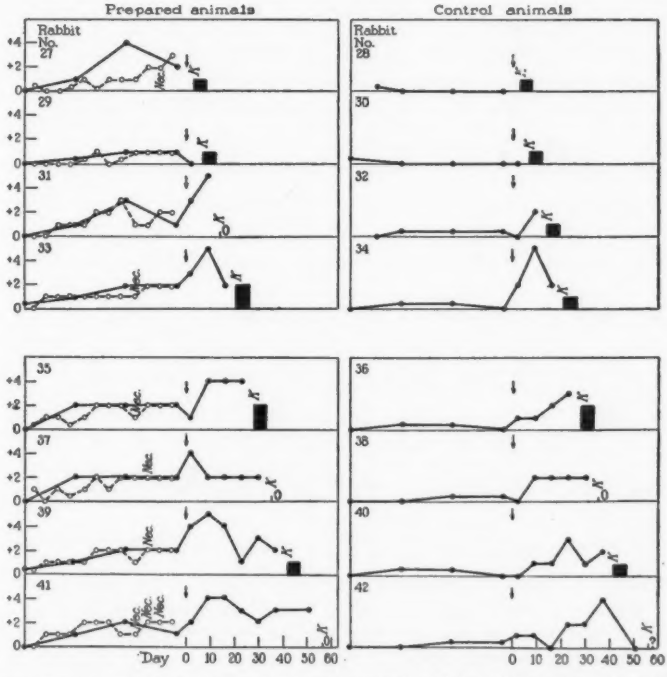
EXPERIMENT 2

In Experiment 2 the suspension of killed bacilli was made by weighing living tubercle bacilli, grinding them and adding saline so that 10 cc. contained 1 mg. of bacilli. It was sterilized in a loosely stoppered bottle in an Arnold steam sterilizer for an hour at 100° C. It was homogeneous on gross examination. Of 12 intracutaneous injections of killed bacilli the first was 0.4 mg., and each of the 11 subsequent injections was 0.2 mg., given at inter-

vals of 5 or 6 days. The reactions to the intracutaneous injections of killed bacilli and to tuberculin are shown in Text-Fig. 2. Eight pairs of white rabbits were used for this experiment, all animals being over 2 kilos in weight.

In this experiment tuberculin tests were made at intervals of approximately 2 weeks. The increase of sensitization following intraperitoneal injection is evident in almost all experiments and in general is more prompt and reaches a higher level in sensitized than in control animals.

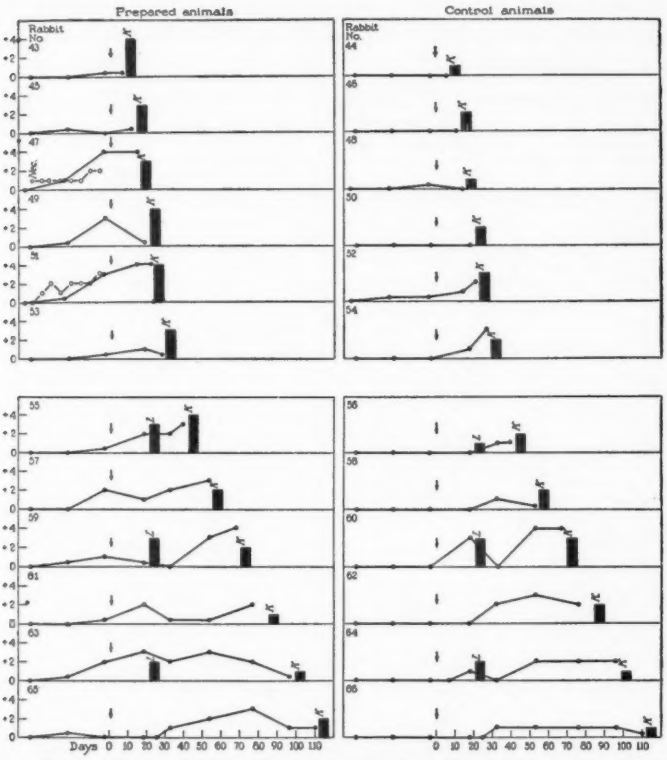
In this experiment the extent of lesions following peritoneal injection is not conspicuously different in prepared and in the control animals.



TEXT-FIG. 2. Lesions of prepared and control rabbits of Experiment 2. The broken lines represent extent of reactions to intracutaneous injections of killed bacilli, the solid lines that of reactions to old tuberculin. The arrow indicates the day on which intraperitoneal injection of killed bacilli was made. The black columns indicate the length of life and the extent of lesions at autopsy.

Experiment 3

The heat killed tubercle bacilli were prepared as in Experiment 2. The sensitizing injections were given in two different ways. Two Havana rabbits (Nos. 47 and 51) were given 2 simultaneous intracutaneous injections of 0.2 mg. of killed bacilli fol-



Text-Fig. 3. Lesions of prepared and control rabbits of Experiment 3. The graphs are drawn as in Text-Fig. 2. The black columns indicate the extent of lesions at autopsy (K), and the extent of lesions observed at laparotomy (L) in 6 animals.

lowed after 5 days by another double injection and 6 single injections of the same quantity at intervals of 5 or 6 days. The other 10 prepared rabbits, which were of gray stock, were given subcutaneously 10 injections each of 0.4 mg. of killed bacilli at

the same intervals. In this experiment a suspension of 10 mg. of killed tubercle bacilli was injected through a blunt pointed needle inserted through the abdominal wall in the left lower quadrant.

Three treated rabbits (Nos. 55, 59 and 63) and their controls (Nos. 56, 60 and 64) were subjected to exploratory laparotomy 21 days after the intraperitoneal injection of killed tubercle bacilli (see Text-Fig. 3). In 1 pair the peritoneal lesion of the sensitized animal (No. 55) was more extensive than that of the control (No. 56), but in both of these animals lesions were more extensive 21 days later when autopsies were performed. In the other 2 pairs sensitized and control animals had equally extensive lesions at the time of operation, but with a longer period between operation and death lesions were less extensive at autopsy than at operation.

In this experiment (Text-Fig. 3) there was, just before intraperitoneal injection, some but not a conspicuous difference in sensitization between the animals prepared by injections of heat killed tubercle bacilli and the controls, but following this injection sensitization in general increased more rapidly and reached a higher level in prepared than in control animals. In this experiment peritoneal lesions were much more extensive in prepared than in control animals.

Lesions Produced by Intraperitoneal Injections of Heat Killed Tubercle Bacilli

Lesions of the Omentum: Dead tubercle bacilli injected into the peritoneal cavity produce more thickening of the omentum in animals that have been given preliminary skin inoculations than in their controls. The omentums of 8 prepared animals (Nos. 3, 5, 7, 13, 17, 33, 47 and 65) were thick, while the omentums of their controls were thin. In only 1 pair was the omentum of the control (No. 32) thick when the omentum of the corresponding prepared animal (No. 31) was not thickened. Prepared animal No. 19 with a thin omentum had 2 controls — 1 with thickened omentum (No. 20) and 1 with a thin omentum (No. 22). In the remaining animals the omentums of prepared animals were approximately the same as those of the controls.

Tubercles were present in large numbers in 13 (45 per cent) of the immunized and in only 2 (7 per cent) of the control animals. Tubercles were present in moderate numbers in 5 (17 per

cent) of the immunized animals and in 7 (23 per cent) of the controls. Tubercles were present in small numbers in 3 (10 per cent) of the immunized animals and in 10 (33 per cent) controls. No tubercles were present in the omentums of 8 immunized and 11 control rabbits.

The omentum of each of the 29 prepared animals and of the 30 controls was examined microscopically. After 3 weeks the control animals are sensitized by the peritoneal injection and hence the animals have been divided into two groups: those that lived 21 days or less and those that lived longer. In the 15 pairs of animals living from 3 to 21 days there were conspicuous differences between the previously treated and the control animals, whereas among the animals surviving from 23 to 112 days controls and treated animals differed little.

Of the rabbits living 3 weeks or less the omentums of 3 prepared animals (Nos. 11, 27 and 31) and of 6 controls (Nos. 6, 8, 12, 28, 34 and 48) showed insignificant cellular reactions. The omentums of 2 of the prepared animals (Nos. 1 and 29) contained loose groups of small undifferentiated monocytes. A scant cellular reaction with accumulation largely of polymorphonuclears, in part necrotic, surrounded by monocytes and in some instances by fibroblasts, occurred in 4 control animals (Nos. 2, 4, 30 and 44). Fully formed tubercles were found in the omentums of 6 prepared animals (Nos. 3, 5, 7, 9, 43 and 45) and in 3 controls (Nos. 10, 32 and 46). By fully formed tubercles is meant compact masses of cells that have assumed the characteristics of epithelioid cells with cell body and nucleus both larger than those of the earlier monocytes. These lesions have the microscopic characteristics of tubercles as found in experimental animals and in man. Necrotic foci are usually present, the necrotic material in some cases containing abundant nuclear fragments, in others containing no remains of nuclei. Acid-fast bacilli are frequently found. It is significant that either loose groups of undifferentiated monocytes or well formed tubercles were present in the omentums of 12 of the 15 prepared rabbits living from 3 to 21 days, but were present in only 5 of the 15 controls living for the same period.

There were 14 prepared animals and 15 controls living from 23 to 112 days and among these animals there was scant difference between prepared and control animals. The omentums of 4 pre-

pared animals (Nos. 37, 41, 61 and 63) and 4 controls (Nos. 22, 38, 42 and 64) showed an insignificant cellular reaction. The omentum of 1 prepared animal (No. 51) and its control (No. 52) contained fully formed tubercles with necrotic foci. In the omentums of 9 prepared rabbits and 10 controls tubercles were regarded as regressive because there was formation of fibrous tissue within them; epithelioid cells had increased in size and central clear areas had appeared in the cytoplasm near the nuclei. Between these large cells with irregular outline were fine fibrils. In the omentums of the animals that lived longest epithelioid cells had decreased in number and giant cells were numerous. A progressive increase in fibrous tissue occurred, sometimes with increased vascularity. Necrosis and acid-fast bacilli were present in the omentums of 9 of the 14 prepared animals of this group. Necrosis and acid-fast bacilli were present in the omentums of 6 of the 15 control animals of this group.

The cellular reaction is greater and the formation of characteristic lesions in the omentum occurs earlier in immunized than in control rabbits. Even more conspicuous is the larger size of the lesions in the immunized and sensitized rabbits. An increased cellular reaction in sensitized animals inoculated with living tubercle bacilli has been repeatedly observed. Nichols [1] injected tubercle bacilli intravenously and found that tubercles developed earlier in the lungs of rabbits that had been previously immunized with avirulent bacilli than in the unprepared controls. Gardner [2] injected guinea pigs intraperitoneally with living tubercle bacilli and found that tubercle formation in immunized animals after 4 to 6 days was in the same stage as in controls after 10 days.

Lesions of Parietal and Visceral Peritoneum: Necrosis evident on gross examination was present at the point of peritoneal injection in 62 per cent of the immunized animals and in 43 per cent of the controls. This necrosis was found especially with the more advanced lesions.

A large number of tubercles was present on the peritoneum of 12 (41 per cent) of the immunized animals but in only 1 (3 per cent) of the controls. Tubercles were present in moderate numbers in 5 (17 per cent) of the immunized animals and in 10 (33 per cent) of the controls. Tubercles in small numbers were found in 7 (24 per cent) of the immunized animals and in 17 (57 per

cent) of the controls. In 5 immunized and 2 control animals of the first 2 experiments, no peritoneal tubercles were present.

Prudden and Hodenpyl in 1891 [3] injected into the peritoneal cavities of rabbits 2 to 3 cc. of a milky suspension of tubercle bacilli killed by the heat of the autoclave and found creamy masses surrounded by epithelial cells and fibrous tissue, but stated that caseation did not occur. Branch and Cuff [4] reported death of guinea pigs following repeated injections of large quantities of killed bacilli into the peritoneum.

Killed tubercle bacilli have been intravenously injected into rabbits by many observers. Prudden and Hodenpyl [3] found tubercle-like masses in the lungs, liver and spleen but none in the kidneys. Masur [5] reported the formation of giant cells without caseation in the lungs of rabbits. Kelber,[6] supported by Baumgarten, maintained that killed bacilli caused a reaction like that produced by a foreign body and no true tubercle formation. Krompecher [7] injected from 1 to 8 cc. of a suspension of killed bacilli and found caseation when the bacilli used were of a virulent strain. Coulaud [8] found tubercles in the lungs of animals injected with suspensions of killed bacilli which were not homogeneous, but found none if the suspensions were homogeneous.

Lesions Outside of Peritoneum: Masses of epithelioid cells occupying wide areas of the tissue were found in about half of the retrosternal nodes that were sectioned and were found both in prepared and in control animals of Experiment 1. Acid-fast bacilli were found only once and doubtless reached these nodes by way of lymphatics.

Groups of epithelioid cells were found in the spleen and liver in more than half of the animals of this experiment, about equally in prepared and in control animals. Groups of similar cells were occasionally found in the lungs but they were not found in the kidneys. These nodules, composed of cells resembling those of tubercles, are evidently caused by dead tubercle bacilli disseminated by the blood stream.

Relation of Sensitization to the Extent of Lesions: It is evident that the procedure used in Experiment 1 gives no adequate insight into the progress of sensitization in animals that have received repeated cutaneous injections of heat killed tubercle bacilli preceding the massive intraperitoneal injection, and has given no

information about sensitization following this injection. In Experiments 2 and 3 sensitization was measured by tuberculin tests repeated at intervals throughout the course of the experiment. It is noteworthy that intraperitoneal injection of heat killed tubercle bacilli is almost invariably followed after a week or more by much increased sensitization, and sensitization reaches a higher level in previously treated animals than that found in control animals that have had no preparation preceding the intraperitoneal injection. Nevertheless it is evident that the control animals as well as the animals prepared by intracutaneous or subcutaneous inoculation are ultimately almost equally sensitized, for after the 3rd week following intraperitoneal injection the difference between the two is insignificant.

In comparing the lesions found in sensitized and in unsensitized animals it should be noted that the two differ conspicuously only during the period shortly after intraperitoneal inoculation. Hence a sharp difference between the two cannot be expected.

Conclusions

When heat killed tubercle bacilli are injected into the peritoneal cavity of rabbits, formation of tubercles and of tuberculous tissue occurs more quickly and becomes more advanced in animals that have received preliminary intracutaneous or subcutaneous injections of killed tubercle bacilli than in their unprepared controls. The difference is greatest 3 or 4 weeks after intraperitoneal injection when the lesions in prepared and in control animals are at their height. After 5 weeks no difference in the two groups is evident and later the lesions in both regress.

The extent of tuberculous lesions is greater in the prepared animals which are both immunized and sensitized than in their controls, but there is no close correlation between sensitization measured by the tuberculin reaction and the extent of tuberculous lesions.

Heat killed tubercle bacilli cause necrosis with the characteristics of caseation both in control (unprepared) animals and in those that have received preparatory injections, but it is more frequently found in the latter. Caseation is in general more advanced in sensitized animals but there is no exact correlation between sensitization and caseation.

When killed tubercle bacilli are injected into the peritoneal cavity, tubercle-like nodules composed of epithelioid cells are often found in the retrosternal lymph nodes, spleen, liver and lungs of both immunized and control animals.

REFERENCES

1. Nichols, Joseph L. Studies on immunity in tuberculosis: an histological study of the lesions of immunized rabbits. Part II. *Med. News*, 1905, **87**, 638–641.

2. Gardner, Leroy U. Studies on the tissue reactions to primary infection and reinfection with the tubercle bacillus. I. A histological examination of the omentum and other subperitoneal tissues. *Am. Rev. Tuberc.*, 1929, **20**, 201–213.

3. Prudden, T. Mitchell, and Hodenpyl, Eugene. Studies on the action of dead bacteria in the living body. *New York M. J.*, 1891, **53**, 697–704.

4. Branch, Arnold, and Cuff, J. R. Tuberculosis. I. Allergic, anaphylactic and immune reactions in guinea-pigs following inoculation with heat-killed tubercle bacilli. *J. Infect. Dis.*, 1930, **47**, 151–170.

5. Masur, Alfred. Zur Kenntniss von der Wirkung todter Tuberkelbacillen. *Beitr. z. path. Anat. u. z. allg. Path.*, 1894, **16**, 256–265.

6. Kelber, Ernst. Ueber die Wirkung todter Tuberkelbacillen. *Arb. a. d. Geb. d. path. Anat. u. Bakt. a. d. path. anat. Inst. z. Tubingen*, 1899, **2**, 378–389.

7. Krompecher, E. Recherches sur le traitement des animaux tuberculeux par la méthode de Landerer et sur la virulence des bacilles tuberculeux. *Ann. Inst. Pasteur*, 1900, **14**, 723–749.

8. Coulaud, E. Bacilles morts et réactions tuberculiniques. *Compt. rend. Soc. de biol.*, 1923, **89**, 1023–1024.

SKIN TUMORS FOLLOWING A SINGLE APPLICATION OF METHYLCHOLANTHRENE IN C57 BROWN MICE *

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Connective tissue tumors have been produced with extremely small amounts of carcinogenic hydrocarbons.[1] A few tumors have followed the application of a single drop of hot tar to mouse skin.[2] It is generally believed that protracted painting with carcinogenic agents is necessary for the development of skin neoplasms. Fieser states that "in order to produce skin tumors in mice with even a highly potent carcinogen in benzene solution some 30 deliberate applications must be made."[3] This statement may be true for many strains of mice. Kreyberg[4] and Bonser[5] have established strains by selective breeding whose skin is especially sensitive to the action of carcinogenic substances.

In investigating the influence of methylcholanthrene on the development of internal tumors in mice the chemical was applied successively at 9 different sites.[6] Papillomas were found at the site of the first painting within 4 to 5 weeks among mice of the C57 brown strain. Investigation of this phenomenon seemed warranted.

C57 brown mice were obtained from the Roscoe B. Jackson Memorial Laboratory where the strain originated in 1925. They have a relatively high incidence of spontaneous internal tumors and less than 5 per cent of mammary carcinoma. The mice were painted when 4 to 5 weeks old with methylcholanthrene 0.5 per cent in benzene. It was applied to the back, from the occiput to the lumbar region, with 2 strokes of a No. 8 camel's hair brush. The animals were not allowed to breed. Sex distribution was equal. They were kept in glass cages and were fed Purina dog chow, water being available at all times.

Forty-four mice were painted with methylcholanthrene. Epilation occurred within 10 days. Ulceration was rarely found. Six-

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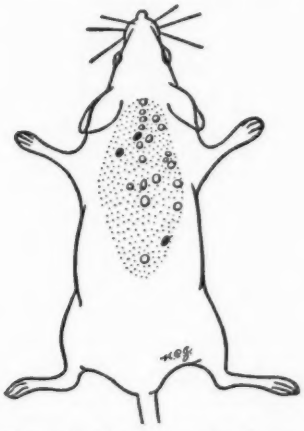
teen of the animals developed a total of 22 papillomas in the painted area (Text-Fig. 1). The first tumor appeared in each mouse between the 31st and 48th days after painting. These lesions had all the gross characteristics of benign skin tumors produced by multiple paintings with carcinogenic hydrocarbons (Fig. 1). They varied from 2 to 8 mm. in diameter, grew rapidly at first and then passed into a stationary period during which their appearance was not altered. Marked variation in the duration of



TEXT-FIG. 1. Distribution of skin tumors in the area painted once with methylcholanthrene 0.5 per cent in benzene. Stippling indicates the painted area; circles represent papillomas; dots indicate carcinomas.

the tumors was noted (Table I). Fifteen of the papillomas regressed completely. The sites of the tumors showed no gross evidence of scarring after the lesions had disappeared. Hair eventually grew in the epilated areas. It was frequently white instead of brown.

Of the 7 papillomas that remained, 3 became malignant and 4 persisted beyond the 225th day after painting. The latter did not change in appearance for more than 100 days, and showed no evidence of progressive growth. The 3 carcinomas occurred in 1 male and 2 female mice on the 228th, 114th, and 124th days respectively (Fig. 2). Diagnosis was confirmed histologically, invasion of muscle being used as the criterion of malignancy (Fig. 3). No metastases were noted but the mice were sacrificed

as soon as clinical evidence of carcinoma was present. In contrast with mice painted twice weekly with methylcholanthrene 0.5 per cent in benzene, these animals remained in good health throughout the experiment.

This reaction of C57 brown mice is probably another instance of genetically determined increase in tissue susceptibility to an ad-

TABLE I

Skin Tumors Following a Single Application of Methylcholanthrene in C57 Brown Mice

Number of mouse	Sex	Papillomas		Duration
		Appeared	Disappeared	
		days	*days*	*days*
1	M	34	102	68
2	M	46	81	35
3	M	38	94	56
4	M	48	76	28
5	M	48	76	28
6	M	41	107	66
		60	105	45
7	F	46	56	10
8	F	39	145	106
9	F	34	76	42
10	F	39	165	126
		42	137	95
11	F	32	105	73
		54	108	54
12	M	26	Carcinoma at 228 days	
		50	72	22
13	F	35	Carcinoma at 114 days	
14	F	31	Carcinoma at 121 days	
15	M	42	Persists at 225 days	
16	M	35	Persists at 225 days	
		54	Persists at 225 days	
		60	Persists at 225 days	

verse environmental stimulating factor. It differs from those produced by Kreyberg and Bonser only in the degree of susceptibility. This is in accord with the concept that carcinoma is not handed down in heredity as such. Cellular susceptibility or resistance to environmental factors is probably the mechanism concerned. A second explanation of the phenomenon would entail a change in the growth and differentiation of skin epithelium by long continued contact with methylcholanthrene. This seems less probable. The fluorescence associated with the chemical cannot be detected in the skin by ultraviolet light 1 week after its application. This is a

crude test and better analytical methods may yield a different result. Furthermore, it is entirely conceivable that a metabolite of methylcholanthrene may initiate neoplasia. Additional experimentation is necessary for the solution of this problem.

The production of skin tumors by a single painting with methylcholanthrene in C57 brown mice appears to afford further opportunity for the investigation of the fundamental physiological changes involved in carcinogenesis. Attempts to affect the biology of the benign tumors may be worth while.

REFERENCES

1. Shear, M. J. Studies in carcinogenesis. I. The production of tumors in mice with hydrocarbons. *Am. J. Cancer*, 1936, **26**, 322–332.

2. Findlay, G. Marshall. The experimental production of cancer by one application of tar. *Lancet*, 1925, **1**, 714–715.

3. Fieser, Louis F. Carcinogenic activity, structure, and chemical reactivity of polynuclear aromatic hydrocarbons. *Am. J. Cancer*, 1938, **34**, 37–124.

4. Kreyberg, Leiv. On the genetic factor in the development of benign tar tumors in mice. *Acta path. et microbiol. Scandinav.*, 1934, **11**, 174–182.

5. Bonser, G. M. The hereditary factor in induced skin tumours in mice: establishment of a strain specially sensitive to carcinogenic agents applied to the skin. *J. Path. & Bact.*, 1938, **46**, 581–602.

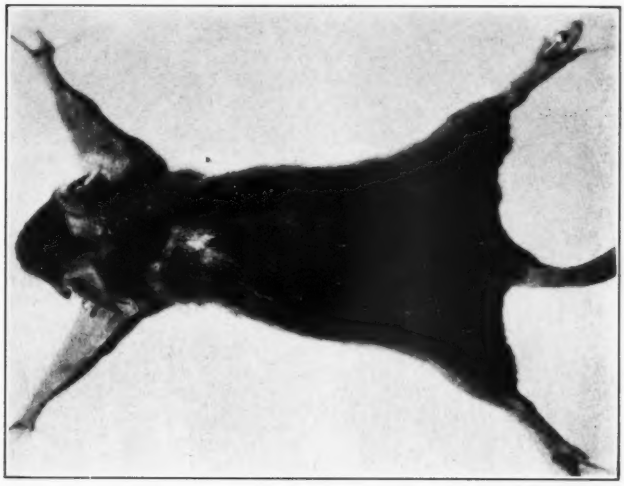
6. Morton, John J., and Mider, G. Burroughs. The production of lymphomatosis in mice of known genetic constitution. *Science*, 1938, **87**, 327–328.

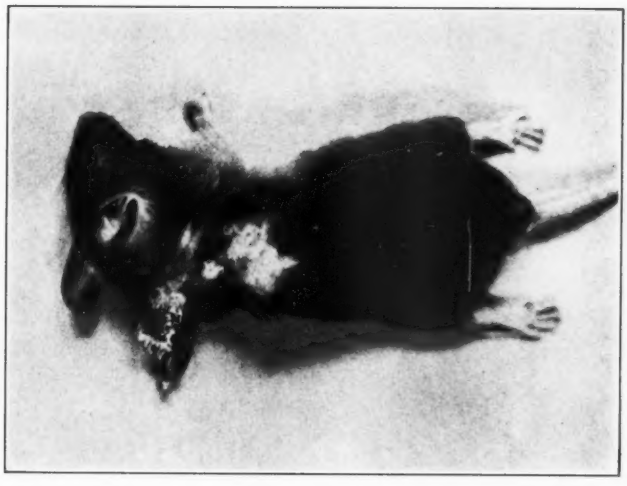
DESCRIPTION OF PLATES

PLATE 53

FIG. 1. Papillomas in a C57 brown mouse painted once with methylcholanthrene 0.5 per cent in benzene. These tumors have been present for more than 225 days.

FIG. 2. Carcinoma in a C57 brown mouse painted once with methylcholanthrene 0.5 per cent in benzene. A malignant tumor appeared 228 days after painting.





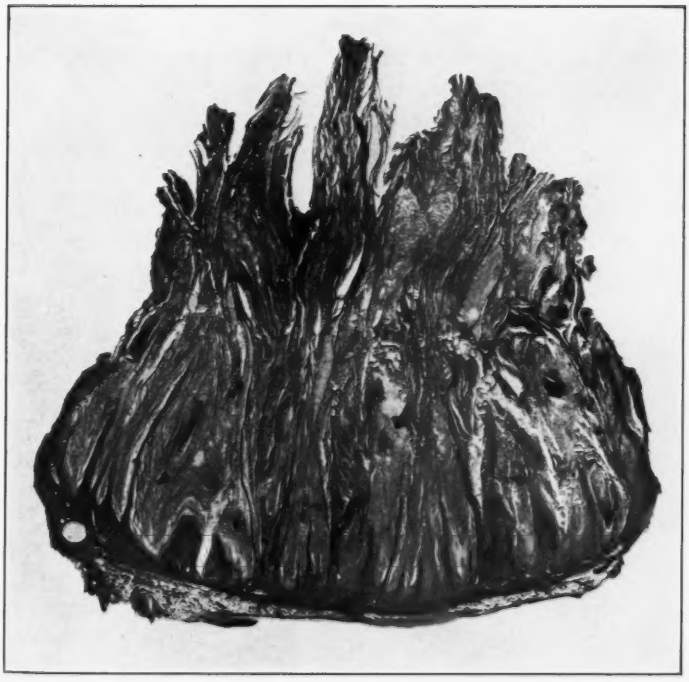
Mider and Morton Skin Tumors in C57 Brown Mice

PLATE 54

FIG. 3. Microphotograph of carcinomatous growth shown in gross in Fig. 2. The tumor cells have invaded the muscle. × 600.

FIG. 4. Microphotograph of a papilloma of the skin arising in a C_{57} brown mouse after a single application of methylcholanthrene 0.5 per cent in benzene. × 10.

3



4

STUDIES ON THE PATHOGENICITY AND CYTOLOGICAL REACTIONS OF THE SUBMAXILLARY GLAND VIRUS OF THE GUINEA PIG *

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The submaxillary gland virus of guinea pigs, according to accounts in the literature, is mild in its effects when inoculated into any part of the body with the exception of the brain.[1–6] In this last location it produces fatal results. The virus we planned to use in a series of experiments failed to act in this manner and fatal results followed injections made subcutaneously, intraperitoneally, into various visceral organs, the submaxillary gland, and also into the brain. It might be assumed from this that we had found a highly virulent strain of the virus. However, the animals that were available for inoculation came from a colony maintained by the Genetics Department of Iowa State College and were proved to be completely free of the disease. In view of these circumstances several problems arose. First, was the difference in behavior of the virus due to a greater virulence than that found by other investigators, or to a greater susceptibility of the guinea pig stock employed. Also, were the histological and cytopathological effects of this virus, which was fatal, different from the reactions described for other submaxillary gland viruses of guinea pigs which were milder in their action. A study of these problems forms the first part of this paper. The latter part deals with stages in the development of inclusion bodies and the time required for each phase. The cytological study was supplemented by use of the ultracentrifuge and the results are compared with those of previous experiments with centrifugation.[7, 8]

MATERIALS AND METHODS

Guinea pigs carrying the submaxillary gland virus were obtained from a dealer in southern Illinois. Uninfected animals

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† We are grateful to the Graduate School of Iowa State College for subscribing to a room at the Marine Biological Laboratory where part of this work was done.

were obtained from the Genetics Department of Iowa State College. Virus for injection was prepared by grinding extirpated submaxillary glands in a sterile mortar which contained a small quantity of sterile sand and Locke's solution. Light centrifugation brought down the heavier particles. The supernatant fluid was injected without filtration; its sterility was tested by inoculation on serum agar and on a broth medium.

Tissues removed from animals were fixed in Zenker's fluid plus acetic acid or in a fixative that permitted subsequent testing for thymonucleic acid according to the procedure given by Cowdry.[9] The stains used were Ehrlich's hematoxylin-eosin and Giemsa's method.

The ultracentrifuge was of the type originated by Henriot and Huguenard and developed by Beams and Pickels.[10, 11] It was made of duralumin and was operated by air from a tank in which the pressure varied from 95 to 120 pounds per square inch.* Revolutions per second of the rotor were determined by matching pitch from a Galton's whistle to the loudest audible note from the centrifuge. It had been observed by Beams [10] that the loudest tone from the rotor corresponds in frequency to the rate of rotation. The results indicated speeds of about 2900 to 3900 revolutions per second from which it was calculated that the centrifugal forces were 300,000 to 550,000 times gravity.

Fresh tissues were placed in the rotor and centrifuged at these speeds for from 30 to 60 minutes, and were then removed and fixed immediately. Uncentrifuged control tissues were prepared as previously described [7] to determine the extent of autolysis due to the time factor involved in centrifugation and to compression effects caused by centrifugation.

I. Experiments and Observations

Results from Virus Passages

Susceptibility of Different Groups of Guinea Pigs: An experiment was designed to ascertain if guinea pigs from a stock of animals that carried the virus (Group I, Table I) were less susceptible than those from a stock in which no virus infection had been present (Groups II and III, Table I).

* We are indebted to Prof. L. T. Brown who kindly permitted us to carry on the centrifugation experiments in the steam and gas laboratories.

TABLE I

Data on Susceptibility to the Submaxillary Gland Virus as Observed in Three Groups of Guinea Pigs

Type of stock used	Age	Number of guinea pig	Duration of life after inoculation into submaxillary gland	Pathological lesions in submaxillary gland		
				Histopathology	Intranuclear inclusions	
					Monocytes	Duct cells
			days			
Animals from a colony carrying the virus	Group I 3 weeks	104	26	Slightly congested	0	0*
		105	17	Congested	0	10
		106	13	Slightly hemorrhagic	1	10
		107	14	Hemorrhagic	7	40
		108	25	Congested	0	100
		109	22	Congested	0	58
			av. 19.5			
Animals from a colony not carrying the virus	Group II 3–4 weeks	110	11	Hemorrhagic and autolyzed	Numerous	(105)†
		112	14	Hemorrhagic and autolyzed	Numerous	(400+)
		113	13	Hemorrhagic and autolyzed	Numerous	(90)
		114	9	Hemorrhagic and autolyzed	Numerous	Unsatisfactory‡
		115	8	Hemorrhagic and autolyzed	Numerous	(108)
			av. 11			
	Group III Adults	116	8	Hemorrhagic and autolyzed	Numerous	Unsatisfactory
		117	10	Hemorrhagic and autolyzed	Numerous	(78)
		118	9	Hemorrhagic and autolyzed	Numerous	(56)
		119	10	Hemorrhagic and autolyzed	Numerous	Unsatisfactory
			av. 9.3			

* Figures in this group represent averages of counts made on two sections.

† Figures inside of parenthesis represent rough approximations after corrections have been made to obtain approximately equivalent size of section, necessitated by extensive tissue destruction.

‡ Unsatisfactory because the severe tissue destruction and autolysis made reliable estimates impossible.

The virus inoculum consisted of 1 part of triturated submaxillary gland and 10 parts of Locke's solution. In preparation for the injection the animals were etherized and an incision was made on the ventral side of the neck to expose the submaxillary glands. A portion of the left gland was removed and fixed. Into the remainder of this gland 0.05 cc. of virus was injected, and into the whole gland on the opposite side 0.1 cc. A total of 0.15 cc. of virus solution was thus injected. All the gland tissues removed before injection proved to be free from intranuclear inclusions. This test proved serviceable as an indication that the guinea pigs did not carry the submaxillary gland virus.

All of the animals died after injection of the virus. They lived for from 8 to 26 days. The average duration of life for young guinea pigs from stock that carried the virus was 19.5 days, for similar young guinea pigs from unexposed stock 11 days, and for adult animals from the same stock 9.3 days. Autopsies made at the time of death revealed a milder tissue reaction in the animals of Group I than in those of Groups II or III. In the animals of Group I there was found an infiltration of the gland and distention of blood vessels. The number of inclusion bodies in the duct cells was relatively small. In the animals of Groups II and III the glands were hemorrhagic and contained large areas that were completely autolyzed. In regions of severe autolysis the duct cells survived but all of the acinar cells disappeared. Inclusions were numerous in monocytes and in duct cells.

The results indicate that the virus used was sufficiently virulent to kill all animals injected, even those that by association in a colony carrying the disease might be expected, from the experience of other investigators, to withstand the action of the virus. On this basis of comparison it appears that the virus employed was more virulent than that used by other workers. The results also demonstrate that guinea pigs from unexposed stock are more easily affected by the action of the virus than those living in contact with the virus. The differences in susceptibility of the animals in Groups II and III, namely between young and adult guinea pigs of the Genetics stock, are slight, but supported by the additional evidence given in Table III, they may be significant. If so, this indicates that adult animals are more susceptible to the disease than young guinea pigs.

Correlation of the Number of Inclusions and the Quantity of Virus: There exists another possible explanation why the virus used in this experiment should appear more virulent than that described previously in the literature. Possibly the animals used as a source of the virus harbored more than the usual amount. Since there exists no direct method for measuring the quantity, the number of inclusion bodies observed was used as a criterion of virus concentration. Numerous investigators have suggested that the inclusion bodies which develop in the duct cells of the submaxillary gland are a fair indication of the presence of the virus, but no effort has as yet been made to correlate the quantity of virus with the number of inclusions.

In Table II, which summarizes the results of the experiment, all guinea pigs that served as a source of virus were shipped from the same dealer but not at the same time. The results obtained indicate how different sources of virus may vary in actual virus concentration. The apparent absence of virus from guinea pigs Nos. 34 to 37, based on the absence of inclusion bodies in 9 sections from a gland of each animal, indicates that failure to find the bodies in a sampled portion of the gland is not complete proof that the virus is lacking. On the other hand, the presence of inclusions is reliable evidence that the virus is present. It is probable that a closer correlation would have been attained between virus concentration and number of inclusion bodies if the number of sections examined had been larger and had been taken from several regions throughout the gland.

Scott [3] is the only investigator who has given data that permit a comparison of results. He injected virus from glands that contained approximately 50 inclusion bodies per section, and although he augmented the pathogenic action of the virus by inoculation of pilocarpine, his animals survived for the duration of the experiment (15 days), whereas those in Groups I and II in Table II, which averaged 39 inclusion-bearing cells per section, died on an average of 7 and 10.4 days respectively. None of the animals survived 15 days.

The reaction of animals in Group IV confirms the conclusion that the virus was more virulent than that employed by Scott; the results obtained with our virus duplicate results obtained by Scott when the concentration of the virus was so low that no inclusion

TABLE II

Relation between the Number of Inclusion-Bearing Cells in the Submaxillary Gland and the Potency of the Virus

Group	Source of virus			Injection in uninfected guinea pigs		Duration of life after injection	
	Number of guinea pig	Number of inclusions per section	Dilution	Number of animals used	Region of injection	Variation	Average
						days	*days*
I	41	(18)*	1:10	4	Submaxillary gland	6 to 8	7
	42	91		2	Brain	4 to 5	4.5
	43	24					
II	44	20	1:10	6	Submaxillary gland	8.5 to 14	10.4
	45	60		2	Brain	4.5	4.5
	46	23					
III	72	14	1:10	3	Submaxillary (1 animal)	14	8
	73	0		3	Brain (2 animals)	Recovered 8	
	74	0					
IV	34	0	1:10	14	Submaxillary gland	All recovered 8 to 11	9.3
	35	0		4	Brain		
	36	0					
	37	0					

* Figure obtained after gross correction had been made so that the size would approximate those that follow.

bodies were observed in the sample sections. Evidence that the virus was present in the animals used as the source of virus in Group IV is proved by the reaction of the 4 guinea pigs which were inoculated intracerebrally.

Dilution Experiments: Since in the previous experiments gland suspensions of high concentrations were employed, it seemed worth while to investigate the action of a diluted virus.

TABLE III

Relation of Virus Dilution to Pathogenesis

Region of inoculation	Number of guinea pig	Dilution of gland (virus) in Locke's solution	Survival after injection		Presence of inclusions in fixed and stained slides	
			Young guinea pigs	Adult guinea pigs	Brain (monocytes)	Submaxillary gland
			days	*days*		
	120	1:20	6		+	
	124	1:40	9		++	
Cerebral cortex. Dose	129	1:80		7	++	
	127	1:80	Survived			−
0.05 cc.	130	1:160	16		−	+
	134	1:320		7	++	
	133	1:320	29*			+
	132	1:320	Survived			−
Subcutaneous,	122	1:20		10		+++
in region of	123	1:20	9			+++
submaxillary	121	1:20	24†			+
gland. Dose,	125	1:40	10			+++
1 cc. (divided	126	1:40	12			+++
between right	128	1:80	19			+++
and left sides).	131	1:160	31*			

* May have died from some cause other than direct action of the virus. Autopsy showed an enteritis.

† Sacrificed on the 24th day; apparently normal at this time.

The data are not adequate enough to determine a sharp end-point in the lethal effects of the virus but indicate that for both intracranial and gland injections of young guinea pigs the end-point is between 1:80 and 1:160. Submaxillary glands pooled from 6 guinea pigs previously injected once or twice in the course of earlier experiments and which had not died as a result of the injections were used as the source of virus. To these were added the glands from an adult guinea pig purchased from the dealer who supplied the guinea pigs harboring the original strain of virus. Emulsions of gland were diluted from 1:20 to 1:320 (Table III). All guinea pigs injected came from the Genetics stock; most of

them were 3 to 5 weeks old and 3 adult animals were also available. Of the 15 guinea pigs, 8 were injected intracerebrally and 7 subcutaneously in the region of the submaxillary glands. In this experiment pieces of gland tissue were not removed prior to the injection, but the subsequent results leave little doubt that the animals were not carrying the virus.

A recent investigation by Markham [6] on the pathogenicity of various dilutions of the virus makes possible a comparison between two sources of virus. He used intracerebral injections in young guinea pigs, and at dilutions of 1:10 the time of death for 4 animals averaged 7.2 days, and no deaths occurred at a dilution of 1:100 or 1:1000.

The data given in Table III lend support to the results reported in Table I, namely that adult guinea pigs may be more susceptible than young guinea pigs as a dilution as high as 1:320 proved fatal in 1 adult animal following intracerebral injection, but in young guinea pigs and at dilutions of from 1:80 to 1:320 death occurred in only 2 of the 4 animals tested.*

Visceral Susceptibility: Thus far the discussion of lethal effects of the virus has concerned inoculations into the brain and submaxillary regions. In the following experiment injections were made into the testicle, kidney, ovary, spleen, liver and abdominal cavity (Table IV). Other animals inoculated intracranially served as controls against which the lethal effects of the virus could be measured.

Glands from 7 guinea pigs furnished the material for injection; 1 animal came from a dealer and the remaining 6 were of the Genetics stock, which were part of the 14 animals included in Group IV, Table II. All animals had recovered from visible effects of the injections made 41 days earlier. For 8 days preceding the time when the animals were to be sacrificed they received subcutaneously 11 injections of pilocarpine hydrochloride; the dosage was gradually increased during the period from 0.4 to 0.7 mg.

As shown in Table IV, the virus was fatal for every animal regardless of the region in which the injection was made. The

* Since these experiments were completed another series of inoculations has been made using animals of various weights and ages and, as before, the full grown animals proved to be more susceptible to the action of the virus than the young animals.

time of death varied from 6 to 11 days. The average duration of life (8.2 days) was somewhat longer than that of 6 days which followed intracranial injection. These results, when compared with those of Groups I and II, Table II, seem to indicate that the visceral organs possess about the same degree of susceptibility to virus action as tested by death dealing effects as that found for the salivary gland.

TABLE IV

Virulence of Virus when Injected in Visceral Organs of Guinea Pig

Animal number	Age	Quantity of 1:10 dilution virus injected	Organ or region injected	Duration of life after injection
	wks.			*days*
135	Adult	0.5 cc. in each	Testicle	7
137	Adult	0.2 cc.	Kidney	6
		0.1 cc.	Ovary	
138	7	0.2 cc.	Kidney	7
		0.1 cc.	Ovary	
		0.05 cc.	Suprarenal	
140	5	No record	Spleen	7
144	7	1.0 cc.	Liver	11
145	6	1.0 cc.	Liver	8
143	Adult	1.0 cc.	Intraperitoneal	10
141	6	1.0 cc.	Intraperitoneal	11
142	7	1.0 cc.	Intraperitoneal	7
				av. 8.2
146	5	0.05 cc.	Brain cortex	7
150	6	0.05 cc.	Brain cortex	6
151	7	0.05 cc.	Brain cortex	5
				av. 6

From the limited data available there is no evidence that one portal of entry among the abdominal organs offers greater resistance to the spread and action of the virus than any other.

As a secondary problem in connection with this experiment, the effect of pilocarpine proved interesting in that there was no apparent increase in the virulence of the virus or the number of inclusion bodies. Scott [3] found that the number of inclusions increased from 50 to as many as 312 per microscopic field, with an average of 203. There was, however, this difference in his procedure and ours; his treatment with pilocarpine began close to the time of the virus inoculation while ours was done after recovery was complete. The different results obtained by these two methods of

treatment confirm his opinion that stimulation of the gland while the virus is in action influences the susceptibility of the cells. In addition, it also indicates that the persistence of infection is probably not due to reinfection.

Brain to Brain Passage: Another minor problem of this experiment was the effort to make brain to brain passages as tried by Cole and Kuttner,[1] Andrewes,[4] and Hudson and Markham.[12] It was thought that the greater virulence of the virus with which we were working might give results different from those reported in the literature. Portions of brains from 3 guinea pigs that died of the disease on the 8th day were inoculated intracranially into 3 adult guinea pigs. All 3 animals survived and were reinoculated intracranially after 17 days with the same virus used for the animals in Group IV, Table II. All survived the reinoculation and so probably had received sufficient virus in their previous intracranial inoculation to confer immunity. These results indicate that the virulent virus behaves in the same manner as the viruses of less pathogenic effects, as reported by other investigators.

Observations

Symptoms: The behavior of the animals following intracerebral inoculation was different from that observed after inoculations made into the visceral or into the submaxillary gland regions. Acute intracerebral infections, which were fatal in 5 to 8 days, usually presented certain nervous reactions such as increased salivation, staggering, loss of equilibrium, incoordination of movements, weakness, or even paralysis of the posterior extremities. Certain animals showed pedaling or convulsive movements prior to death. The variations in temperature were similar to those described by Cole and Kuttner,[1] and Markham [6] in that following injection of the virus there was a protracted high temperature which dropped to a subnormal level about 24 hours before death. Acute infections after inoculation of the virus into the submaxillary gland, the adjacent region, or into the peritoneal cavity or the abdominal viscera, produced death in from 7 to 12 days but failed to elicit nervous symptoms. The usual reaction was that of general depression and loss of appetite after 4 to 6 days. In this condition the animal was not alert, its eyes were often closed, and there was frequently a marked swelling of the submaxillary region

which in some cases seriously impaired respiration. The temperature in different animals was somewhat elevated but never reached the levels caused by the intracerebral inoculations. Death ensued in these animals rather quickly and unexpectedly in comparison with the animals inoculated intracerebrally.

Histopathology: The lesions produced by intracerebral inoculation of the virus were similar to those described by many investigators. They consisted of a peracute to acute inflammation of the meninges characterized by extensive hyperemia and monocytosis. The peracute lesions were encountered in the animals that died in from 4 to 6 days after inoculation and consisted of invasion by considerable numbers of macrophages with formation of large giant cells. When death occurred after 6 days there was a decrease in congestive changes but an increase in the number of infiltrating monocytes. There was present also an increasing microglial proliferation in regions of the brain cortex adjacent to the acutely inflamed regions of the meninges. This microglial proliferation was first noted on the 7th day, becoming pronounced on the 9th to 11th days.

Inoculations of the virus into the submaxillary gland or adjacent subcutaneous tissue produced extensive and macroscopically congestive, hemorrhagic and degenerative changes in the whole region. Microscopically there was abundant infiltration with monocytes accompanied by hyperemia, extensive extravascular accumulations of blood, and marked tissue destruction. In addition, macrophages and small giant cells were seen, but to a lesser extent than in the more acute brain lesions already described. Destruction was especially marked in the submaxillary gland tissue. The first gland cells to be destroyed were those of the acini, while the duct cells seemed to be more resistant and frequently persisted in regions where all of the surrounding cells had been completely autolyzed. If the animal survived an acute attack the severely damaged gland regenerated and mitotic figures could be found. Regeneration was almost complete by the 21st day after infection.

Virus inoculated into the visceral organs produced inflammatory reactions similar to those seen in the submaxillary gland. There was extensive degeneration of the inoculated organ, frequently to such an extent that it was difficult to distinguish the original cells

from those that had migrated into the organ, which were mostly monocytes. In addition to these changes there was usually a more or less severe inflammation of the peritoneal cavity. Giant cells were usually found in most of the visceral organs studied.

After inoculation of virus into one part of the body there frequently occurred a reaction in organs distant from the site of injection, for example an injection into the brain of an animal caused a reaction in the lymph glands of the submaxillary region after 5 days, and 8 to 12 days following an injection into the submaxillary gland an inflammatory reaction was present in the spleen and other visceral organs. The reaction in the secondary location was always less severe than that at the original site of inoculation.

Cytopathology: The main pathological change observed was the formation of inclusion bodies. In addition to descriptions of inclusion formation in the submaxillary duct and acinar cells the literature includes the following references on inclusion formation: cells of the testis, tongue, and lung (Cole and Kuttner [1]); mesodermal (mesenchymal) cells of the guinea pig fetus (Markham and Hudson [13]); and fibroblasts, endothelial cells, smooth muscle and kidney cells (Pearson,[14] and Kuttner and T'ung [5]).

Inclusions were usually formed at the site of inoculation of the virus, although they were also found frequently in tissues distant from the area inoculated. Of the latter tissues, the submaxillary duct cell was more frequently affected, but the liver cells also tended to develop inclusions, although of a type different from those formed in the gland.

The cells affected directly through local administration of the virus included monocytes, macrophages, endothelial cells and lymphocytes of the spleen and lymph glands, cells of the tubules of the kidney, the thyroid gland, a few nerve cells of the brain cortex, the theca cells of the ovary, interstitial and possibly some germinal cells of the testicle, epithelial cells of the epididymis, and a few cells of the adrenal. The lung, pancreas and mammary glands were not studied. The monocytes constituted a particularly interesting aspect of the disease in that they increased greatly in numbers in whatever tissue was inoculated, infiltrating not only at the site of inoculation but many other distant areas. The monocytes carried inclusion bodies through the course of the clinical

disease up to the 11th day in animals inoculated intracerebrally and up to the 14th day after inoculation in the submaxillary gland.

The wide distribution of these inclusion-bearing cells distant from the site of inoculation is given in Table V, but whether the inclusion is a sign of the presence of virus in all the tissues of the guinea pig during the 5 to 12 days of the disease, or whether it represents a migration of monocytes already carrying the inclusions could not be definitely stated from our experiments until further relations are established between the virus and inclusion-bearing monocytes. Kuttner and T'ung,[5] however, have shown

TABLE V

Occurrence of Inclusion-Bearing Monocytes in Regions Distant from Point of Injection

Animal number	Region inoculated	Death and removal of tissues	Organs with inclusion-bearing monocytes
		days	
52	Submaxillary	8	Spleen, liver
54	Submaxillary	11	Kidney, suprarenal
122	Submaxillary	10	Liver
126	Submaxillary	12	Liver
140	Spleen	7	Submaxillary gland, liver, stomach
144	Abdominal cavity	11	Submaxillary gland?
147	Brain	5	Lymph gland (submaxillary area)
150	Brain	6	Lymph gland (submaxillary area)

that at the 10th day following intracerebral inoculation the virus is present in many tissues of the body.

The evidence seems to indicate that the inclusion-bearing monocyte is not a criterion of the presence or amount of the living virus in that inoculation of large numbers of such cells in brain to brain passages and the tissue culture experiments of Andrewes [4] have failed to keep the virus alive. Andrewes came to the conclusion that in tissue culture survival of the virus is established but multiplication does not occur. On the other hand, the monocyte may possibly engulf and thereby destroy the virus.

II. CYTOLOGY OF INCLUSION BODY FORMATION

This study of inclusion body formation in the guinea pig and a similar study undertaken on the cytology of fox encephalitis have indicated that the response of the chromatin is a more reliable measure of the age of the inclusion than is the size of the

inclusion body. Intranuclear inclusion bodies must increase in size from a small beginning, but it would seem that this growth is not uniform if compared with changes that occur in the nucleus.

Development of Inclusion Bodies in Monocytes, Macrophages and Giant Cells

The "early" stage of inclusion formation, judged by the criterion of nuclear reactions, is a slight pulling away from the inclusion body of the chromatin granules and their connecting linin network. In the earliest stages no appreciable halo exists between the inclusion body and the chromatin granules. This condition is rare and usually a halo has developed, as shown in Figures 1 and 2. In both figures the nuclear reaction has progressed to about the same degree in relation to the inclusions. The condition in Figure 1 is more typical in that the inclusion body is single and small. In Figure 2 an extreme variation from the typical condition is illustrated.

Continued margination results in retraction of the chromatin granules toward the nuclear membrane (Fig. 3). As a rule the granules of chromatin which have not yet reached the margin appear still definitely discrete and separate, as do the granules of chromatin in the earlier stages, or as those in the normal resting nucleus. Probably the only pronounced difference is an increase in amount of oxychromatin in the inclusion-bearing cell.*

As margination proceeds the chromatin which reaches the nuclear membrane first fuses into a seemingly homogeneous mass. Upon this lie groups of chromatin granules which have aggregated together in irregular clumps (Fig. 4). The chromatin in contact with the nuclear membrane becomes inconspicuous but apparently does not diminish in amount. Such a stage as this has been designated as "intermediate." The inclusion body in Figure 4 is

* Oxychromatin is a term difficult to define because we have little definite knowledge concerning its chemical nature. In stained preparations it is usually regarded as an acidophilic substance. It has a negative Feulgen reaction. There seem to be two types of acidophilic material in the nucleus: first, a portion that behaves like the basichromatin, in that it mingles with it, has nearly the same specific gravity, and is readily pulled away from the nuclear membrane during centrifugation; and second, a portion of viscous nature which during centrifugation stretches into fine strands when pulled by the heavier parts of the nucleus. Wilson [35] is of the opinion that oxychromatin and basichromatin are two phases of the same substance.

about as large as that in Figure 7, which illustrates again how variable their size may be in relation to the nuclear reaction.

In the particular cell from which Figure 4 was made the inclusion body revealed two parts which, structurally and by the staining reactions, are distinct from each other. One is a spherical portion with an affinity for acid dyes, such as eosin used in the hematoxylin and eosin stain or in Giemsa's stain. This body is relatively homogeneous in structure. The second is a darker staining portion which usually contains spherical vacuoles. The usual relationship of the two portions is shown in Figures 4 and 6, in which the darker vacuolated mass forms a cap at one side of the acidophilic body, but occasionally the bodies are completely separated (Fig. 5 ABIN and AIN). This latter arrangement emphasizes the fact that the bodies are not merely the result of an optical phenomenon. However, evidence from staining procedures makes it seem probable that both bodies are of similar origin, although they stain differently. The darker body is not colored with the hematoxylin in the same way as is chromatin, instead it appears gray or slate colored. Feulgen's reaction alone does not color the acidophilic body but does color lightly the "dark body." When the Feulgen reaction with a light green counter stain is employed the acidophilic body stains green and the "dark body" stains gray or grayish blue. It is interpreted from these reactions that the dark inclusion body is fundamentally similar to the acidophilic body and that it has an affinity for the light green stain to the same extent, but in it there is dissolved chromatin, which gives it its slightly positive Feulgen character; the red and green dyes mixed give the slate color. Likewise, the combination of blue and orange colors from the hematoxylin and eosin stain gives a bluish slate color. In order to have a term for each type, one has been called the acidophilic inclusion and the other the acidobasophilic inclusion. Why one inclusion body should have an affinity for chromatin and the other should not finds no conclusive answer at present. Some meager evidence points to a difference in age, the one that contains chromatin being the older. Reason for this suggestion comes from the basophilia observed in many inclusion bodies under conditions when they are known to be old. Inclusions of both types are found not only in monocytes but also in young intranuclear inclusions of the submaxillary gland ducts.

The process of margination of the nuclear chromatin continues from that represented by Figure 4 and relatively few separate granules remain still distinguishable, a late "intermediate" state (Fig. 5). Margination ultimately goes to completion (Figs. 6, 7 and 8) which has been designated as a "late" state. The nucleolus may lag behind in this process but later reaches the nuclear membrane (compare Figures 6 and 8). At this stage it was expected from a previous study on fox encephalitis that the marginated chromatin would break up and aggregate into masses, but this was not the case with the monocytic intranuclear inclusions of the submaxillary gland virus of the guinea pig. In studying the final fate of these cells only those that were surrounded by living and unautolyzed cells were considered in order to eliminate, if possible, all non-specific extracellular toxic effects as indirect causes of cell changes. The number of cells which fulfilled this precautionary measure was relatively few. Only very rarely was autolysis seen in monocytes located in the midst of living cells. The process of autolysis when observed is similar to that found in giant cells (Fig. 10); the chromatin liquefies, spreads throughout the nucleus and obscures the inclusion body. Nuclear fluids disappear and ultimately the nuclear membrane shrinks against the inclusion body. This type of autolysis is similar to that observed by Lucas and Cowdry in the nuclei of liver cells in healthy rats. The rats were killed by a blow on the head and their bodies put in the incubator at 37° C. for various periods of time. The condition in Figure 10 is somewhat similar to that shown by the nuclei of a rat's liver after the animal had been dead for about 18 hours.

Since so few autolyzing monocytes were found in the meninges of the brain, search was made among other tissues, such as the spleen, cervical lymph glands and several visceral organs, to find, if possible, where these autolyzing cells were more abundant. They were not present in any of these tissues in numbers sufficient to make it seem likely that their fate was the simple autolysis for which we had been looking. Attention was drawn ultimately to the giant cells. In the literature has been described the transformation of monocytes and macrophages into giant cells. There was evidence that these two cell types were probably involved in the formation of giant cells in our material. Autolysis of giant cells

was found more frequently than the autolysis of monocytes and it was concluded, therefore, that some monocytes, at least, undergo autolysis after they become giant cells rather than in the mononuclear condition.

As far as we are aware this is the first time that the occurrence of giant cells has been noted after the injection of submaxillary gland virus. They do not appear in every preparation where there is an infiltration of monocytes. They may be found as early as 4.5 days, as well as in later stages when the acute congestive changes have subsided.

Classification of giant cells, particularly the separation of epithelioid giant cells (Langhans' giant cell) and foreign body giant cells is difficult without the use of vital dyes. Doan, Sabin and Forkner,[16] and Forkner [17] noted a structural difference, however, in that the nuclei of the epithelioid giant cell are arranged at the periphery of the cell, whereas the foreign body giant cell has its nuclei scattered through the cytosome. Another distinction for the separation of the two types is that the epithelioid giant cells are thought to be formed by amitotic divisions of monocytes, whereas foreign body giant cells form from the fusion of macrophages.

Both types of giant cells were frequently found in our material but the epithelioid giant cell was perhaps more in evidence because the arrangement of its nuclei made it more conspicuous (Figs. 10 and 11). Lobulation of nuclei was more abundant in smaller than in larger cells. There were many cells with deep constrictions among the lobes which presumably were in the process of dividing into separate nuclei. In many cases two large lobes would be held together only by a small strand. This type of lobulation was observed by Unna,[18] Tyzzer,[19] and Lipschütz [20] following infection with herpes, varicella and variola, and vaccinia, and has been called by these authors "ballooning degeneration," the end result of which is the production of a cluster of nuclei in the center of the cell.

Foreign body giant cells were identified by their scattered nuclei and the remains of phagocytozed débris in the cytosome. Whether fusion of cells which is characteristic of foreign body giant cells occurred in our material is difficult to establish with certainty. There were frequently observed, however, cells pressed into the

side of a giant cell, and in some cases part of the cell wall between the two seemed to be absent.

Certain small giant cells seemed to take their origin from the endothelium of blood vessels in congested regions and they were found in numbers within blood vessels.

Although there may be found both types of giant cells, yet there are all intermediate grades so that it is not certain to what extent we are dealing with valid foreign body giant cells or with epithelioid giant cells in which the monocytes from which they were formed were modified as described by Carrel and Ebeling,[21] and Lewis,[22] in the transformation of monocytes to macrophages.

If giant cell formation represents the ultimate fate of a portion of the monocytes with inclusions, and since autolysis is frequently observed in these cells, it may explain the rarity of autolysis among mononuclear monocytes. The process of autolysis in the nuclei of giant cells is similar to that for the monocyte, namely liquefaction of the chromatin, loss of nuclear substance, and shrinkage of the nuclear membrane (Fig. 10).

In some of the giant cells there were nuclei without inclusion bodies or the inclusions present were in different stages of development, but in the majority of cases all of the nuclei in one giant cell were in the same stage of development, namely, complete margination. There is the possibility that the giant cells and monocytes do not always deteriorate and die as a direct result of their abnormal states; Forkner,[17] for example, is of the opinion that giant cells may later split up into component cells but there has been no evidence for such a process in our material.

Whether the inclusion bodies of monocytes or giant cells always undergo autolysis or persist after the cell disintegrates has an important bearing on the relation of inclusion bodies to the virus agent. The inclusions in Figure 10 would seem to show disintegrative changes as described by Ivanovics and Hyde [23] for virus III in tissue culture. On the other hand, old inclusions entirely free from their cells could be definitely identified in the intercellular spaces and such bodies have been phagocytozed by other cells (Fig. 8 ININ).

The time involved in the various phases of the process of inclusion formation and development in the monocyte is not known since all stages appear simultaneously in one preparation. The

relative time may however be estimated from the relative abundance of cells in different development stages. Early stages (Figs. 1, 2 and 3) and autolytic stages (Fig. 10) are few in comparison with the intermediate and late stages (Figs. 4, 6, 9 and 11) which seems to indicate that early and autolytic stages probably change rapidly and that intermediate and late stages are more enduring. It is also probable that the whole cycle of inclusion-bearing monocytes is relatively short in comparison with the life of infected duct cells of the salivary gland since the former shows late stages and autolysis at the end of 4 or 5 days, whereas development of late stages in the latter requires about 2 weeks.

Development of Inclusions in Duct Cells of the Submaxillary Gland

A reconstruction of the sequence of events in the duct cells is attended with much greater certainty than in the monocytes because the development of inclusion bodies in the duct cells is synchronized. Because of this it is possible to gain a fairly accurate estimate of the time required for the development of inclusion bodies "*in vivo*."

Kuttner,[2] Andrewes,[4] Kuttner and T'ung,[5] and Hudson and Markham [12] noted that the development of inclusion bodies was usually delayed until about the 10th to 15th day after inoculation. The occurrence of an initial delay has been observed in our material except that inclusions appeared earlier. They were never absent in any animal that died later than the 6th day.

The nuclear chromatin reacts to the presence of a newly formed inclusion body in about the same way as in the monocyte, in that there is a drawing of the chromatin away from the inclusion body. At first this action is slight (Fig. 16, 7 days, and Fig. 17, 11 days). In the "early" stages strands of oxychromatin still cross the developing halo and the chromatin granules are separate and discrete. The process of margination goes approximately to completion (Fig. 18, at the 11th day) which is still considered as an early stage in the formation of inclusions in the duct cell whereas it would be considered "intermediate" to "late" in the monocyte. It should be emphasized that usually more than one stage of development as given in the figures could be found on a single slide, but there were definite limits to this variation so that the

stages of development could be fairly closely correlated with the length of time the animal survived after inoculation.

Chromatin margination is similar to that observed in the monocytes, but in the latter autolysis seems to be the next stage in the cycle, whereas in the duct cell a different reaction occurs. The chromatin, after it attains the margin, or even before that stage is reached (Fig. 19, 13 days, and Fig. 22, 17 days) breaks away and moves toward the inclusion body. This is considered as the "intermediate" stage in nuclear reaction; it includes the period when the amount of centrally migrated chromatin is still small and particulate, and more or less uniformly distributed around the inclusion body. With increasing age more of the chromatin accumulates (Fig. 23, 17 days, and Fig. 20, 21 days). Then follows a slight clumping of chromatin granules which ultimately goes as far as illustrated in Figure 21, a cell taken from a section of tissue from a stock animal used as a source of virus. The clumping of the chromatin, which has moved centrally, and its almost complete absence on the margin is designated as an "old" stage. In none of the cells observed, however, were all the granules clumped together. As a result of the central migration of chromatin the nuclear margin becomes nearly bare.

The description of the old stages agrees with the previous studies made by one of us,[8] with the exception of one point. In the experiments published in 1936 it was stated that all of the basichromatin moved centrally. This appeared to be the case when hematoxylin and eosin were used as a stain, but after the Feulgen reaction was applied (Figs. 22, 23 and 24) it was discovered that a small amount of finely dispersed basichromatin remained mingled with the oxychromatin on the nuclear membrane.

When these criteria of age are applied to the colored illustrations of submaxillary gland duct cells by Cole and Kuttner,[1] Figure 2 would be regarded as a young stage, as they suggested, and Figure 1 an intermediate stage.

The final fate of inclusion-bearing cells could not be established definitely. In many cases they were apparently extruded into the lumens of the ducts and this was observed at various stages of inclusion formation. Autolysis was observed in only a few of the older cells, but this occurred only in regions where tissue destruc-

tion was pronounced. When it did occur it followed the same cycle as revealed in degenerating monocytes, namely liquefaction of the chromatin, loss of nuclear fluids and shrinkage of the nuclear membrane against the inclusion body (Fig. 25).

A characteristic cellular reaction recorded in most of the literature and also found in our studies is the occurrence of cell and nuclear hypertrophy. The cell in Figure 18 (11 days after inoculation) already shows marked hypertrophy. This effect is continued for at least 2 weeks after the first appearance of intranuclear inclusions (compare Fig. 18 with Fig. 24 from an animal 24 days after inoculation).

The inclusion body during its formation often shows the same two types of parts found in monocytes; one stains gray (acidobasophilic inclusion) with hematoxylin and eosin, and the other pink (acidophilic inclusion). The same explanation of formation holds true, namely that the gray body contains a certain amount of dissolved chromatin. As the inclusions become older the amount of dissolved chromatin increases and old inclusions nearly always stain darkly.

The cytoplasmic inclusions were probably first observed by Jackson [24] who reported them as parasitic organisms. Pearson [14] and Andrewes [4] later studied them. Andrewes considered them secondary to nuclear inclusions, while Pearson proved them to be faintly Feulgen-positive, a fact later confirmed by Markham.[6] Similar bodies have been observed in the submaxillary glands of man by Goodpasture and Talbot,[25] and by Farber and Wolbach.[26]

In our study the cytoplasmic inclusions are scarcely visible when stained with hematoxylin and eosin after fixation in Zenker's acetic acid solution, while the stain is somewhat better after fixation in corrosive sublimate plus acetic acid. They stain blue with Giemsa's stain. The results are best after the Feulgen reaction, by which method the inclusions are the only cytoplasmic elements that give a positive reaction. The cytoplasmic inclusions proved to be different from the intranuclear ones in that they had an affinity only toward the Feulgen reaction and not toward the light green counterstain which indicated a distinct difference between the two inclusion bodies. The Feulgen-positive character of the intranuclear inclusion is a secondary quality, whereas it is a primary one for the cytoplasmic inclusion. Haagen [27] reports that

the cytoplasmic inclusion of vaccinia virus is Feulgen-positive but is of the opinion that in this case the Feulgen-positive material is derived from the basichromatin of the nucleus.

The development of cytoplasmic inclusions lags behind that of nuclear inclusions, as already reported by Andrewes.[4] They appear on or before the 17th day after inoculation (Fig. 22), which is 6 to 10 days after the first appearance of the intranuclear inclusions. The cytoplasmic inclusion appears first as a small granular body, but in the same slide from which Figure 22 was chosen, older stages were found in which the number and size of these bodies had increased, as shown in Figure 23 CIN. In this particular cell the particles which aggregated to form the inclusion bodies were sufficiently separated so they could be distinguished individually. It proved difficult to measure the diameter of a single particle because Feulgen's technique does not stain very strongly a particle so small, but their diameters were estimated to be about 400 $\mu\mu$. When the inclusion bodies are older they form compact masses and individual particles are no longer visible (Fig. 24, 24 days), and the number of inclusion bodies increases also.

When these cells autolyze (Fig. 25) the outline of the cytoplasmic inclusions becomes less definite, but the reaction may be due more to disruptive and disintegrative changes in the cytoplasm than to autolysis within the inclusion body itself.

These cytoplasmic inclusions are usually observed at the lumen side of the cell, which usually presents the larger amount of cytoplasm. This agrees with observations by Pearson.[14]

The rate at which intranuclear and cytoplasmic inclusion formation takes place may be summarized as follows: intranuclear inclusions may appear as early as the 6th day after inoculation but probably they develop later in the animals less acutely affected. The early steps of margination proceed rapidly and are usually found with the beginning stages. The migration of the chromatin toward the inclusions (intermediate stage) begins about the 13th day after inoculation and extends up to the 17th day. The old stages, characteristic of the condition found in stock animals, appear on the 21st day after inoculation and are abundant by 24 days.

The cytoplasmic inclusions first appear when the nuclear cycle is half completed, namely about 17 days. They develop more

rapidly than the nuclear stages so that at 24 days they are as numerous as they are in stock animals.

It has been mentioned that intranuclear inclusions develop in many other tissues of the body, but the sections prepared were not sufficiently abundant to allow an accurate determination of the sequence of developmental stages. Those in the liver cells were given particular attention because of their similarity to the "chemical inclusions" reported by Olitsky and Harford.[28] * The inclusion body in the liver cells is hyaline, stains with acid dyes, although rather lightly, and the particular feature which made them appear similar to those reported by Olitsky and Harford is the apparent compatibility of the chromatin with the inclusion body and the absence of a clearly defined halo around the inclusion body (Fig. 15). The chromatin in the cells is only very rarely completely marginated; usually there is little or no margination. The nuclear reaction in the liver cells is similar also to that illustrated and described by Blackman [29] in kidney cells following lead poisoning.

Effects of Ultracentrifugation on Inclusion-Bearing Cells

Earlier studies on the centrifugation of herpes inclusions in cells of the rabbit cornea demonstrated that the inclusion body was lighter than either the nuclear sap or the chromatin.[7] The centrifugation of glands from stock guinea pigs infected with the submaxillary virus failed to separate the chromatin from the intranuclear inclusion and both were carried to the centrifugal pole.[8] It was hoped that by using early stages before the chromatin joined the inclusion body the relative specific gravity of the inclusion body could be determined and compared with that of herpes.

In the present investigations the effects of centrifugation on duct cells of different ages, on monocytes and on lymphocytes were studied. The movement of the chromatin in both lymphocytes (Fig. 12) and monocytes (Figs. 13 and 14) is independent of the degree of margination. The partially or totally marginated chromatin moves to the centrifugal pole while the inclusion body remains suspended in the nuclear sap. When completely marginated the chromatin seems to slide along the nuclear membrane

* Dr. Olitsky and Dr. Harford kindly sent us a stained slide and a block of tissue from their experiments in order that we might make more direct comparisons.

to reach the centrifugal pole. The number of cells available for study were too few to give any idea whether the inclusion body, if centrifuged longer, would have moved upward or downward through the nuclear sap in which it was suspended. In several cells two types of intranuclear inclusions were distinguishable. After centrifugation (Fig. 13), however, no consistent orientation could be observed which would indicate that one was heavier than the other.

In the submaxillary gland duct cells, 10 days after inoculation when the intranuclear inclusion bodies are beginning to form and when little or no chromatin has moved on to the inclusion body, centrifugation concentrated the basichromatin and oxychromatin at the centrifugal pole (Fig. 26). The inclusion body follows the chromatin, and thus demonstrates that it is lighter than the chromatin but heavier than the nucleoplasm.

When the stage of development has been reached in which the chromatin particles migrate to the inclusion body, centrifugation carries the loose marginated chromatin to the centrifugal pole but it does not separate that which has joined the inclusion body (Fig. 27, 14 days).

By the time cytoplasmic inclusions are numerous and the nuclear changes are well advanced, centrifugation causes the movement of the inclusion and its adherent chromatin to the centrifugal pole where it may produce a bulging of the nuclear membrane, as in Figure 28. In addition, the evidence is fairly conclusive that the cytoplasmic inclusions are heavier than anything else in the cytosome, and perhaps as heavy as the nucleus. In nearly every cell studied they were located as close to the centrifugal pole of the cell as the nucleus would permit. In some cases the nucleus and its inclusion body touched the cell wall and under these conditions the cytoplasmic inclusions were located at the side of the nucleus, but near the centrifugal pole. The high relative specific gravity of the cytoplasmic inclusion perhaps has some relation to the Feulgen-positive nature of the material of which it is composed.

III. Discussion

Many points of limited interest were discussed under the experimental data. Several of the larger problems on which these

investigations have a bearing are the origin and fate of inclusion bodies, and the viability of the cells which bear them. The significance of the centrifugation studies will be discussed.

Origin and Fate of Inclusion Bodies

Nearly all structural parts of the cells have been suggested as the source or origin of inclusion bodies. In addition they have been considered to be aggregations of virus particles. As an example, Negri bodies have been suggested to be of neurofibrillar and mitochondrial origin by Goodpasture,[30] from Nissl substance by Covell and Danks,[31] and Nicolau and Kopciowska.[32] The origin of vaccinia bodies has been stated to be from basophilic material of the cytoplasm (Cowdry [33]), from nuclear material passed from the nucleus to the cytosome (Taniguchi *et al.*,[34] and Haagen [27]), from accumulation of virus elementary bodies (Paschen,[35] Goodpasture, Woodruff and Buddingh,[36] and Burnet and Andrewes [37]). In the formation of intranuclear inclusions it has at times been suggested that the plasmosome hypertrophies to form the acidophilic body. In this regard it is probably worthwhile to present evidence that this is not the case in the inclusion body formation of either the monocyte or of the submaxillary duct cell. First, a nucleolus is absent in some of the blood cells, namely lymphocytes and small monocytes, so that the acidophilic body could not develop from the plasmosome. In large monocytes (Figs. 6, NL and 8), in endothelial cells, and in nerve cells in which inclusion bodies were formed, the nucleolus was pushed toward the nuclear membrane and showed no affinity for the developing inclusion body. The most convincing evidence, though, comes from the submaxillary duct cell (Fig. 23) and the liver cell (Fig. 15 NL). In the duct cells the plasmosome, which is distinguishable during the margination process, is pushed against the nuclear wall. This is shown well also in the liver cell in which the inclusion body is distinct from the plasmosome. The plasmosome is covered with adherent basichromatin, but the inclusion does not attract the basichromatin in the same way.

Related to the problem of lineage of cell organelles in inclusion formation is the problem dealing with the causes underlying margination, separation of oxyphilic and basophilic chromatin, the time it takes place, and the intracellular movements of other cell

material. The mechanics of margination are apparently the same in the three examples studied closely, namely, the meningeal cells reacting to the virus of fox encephalitis, and the monocytes and the duct cells of guinea pigs under the action of the submaxillary gland virus. There is this interesting difference which may have significance as more material becomes available; the inclusion body of fox encephalitis is Feulgen-positive from its earliest beginning, while that of the submaxillary disease is negative in the early stages. Corresponding to these differences, in fox encephalitis the oxychromatin completely separates from the basichromatin and migrates centrally to apply itself to the surface of the inclusion body while the basichromatin moves to the nuclear membrane. In the submaxillary gland duct cells of guinea pigs both oxychromatin and basichromatin marginate, but after the initial margination spurt is over the basichromatin returns to the acidophilic inclusion body and becomes so closely attached that it has not yet been separated by centrifugal forces over 500,000 times gravity. Thus, in 1 case oxychromatin unites with a basophilic inclusion body, and in the other basichromatin unites with an acidophilic inclusion body.

The question might be raised, why does not the same central movement of chromatin take place in the monocytes as described for the duct cells? It does take place, for example, in Figure 7, but it is found only rarely and never to a greater degree than illustrated. Only one suggestion can be offered as to why it does not go as far in the monocyte as it does in the duct cell, namely that the monocyte does not survive long enough. We do not know the life span of the cells in a congested area, but the complete cycle of tissue changes from inoculation to the period when monocytes have reached a peak in numbers is about 5 days, and in the next 5 days practically all have disappeared. The life span of a single cell in such an area of rapid change is either very short or it changes so completely that its lineage has not been followed. If its life were less than from 3 to 5 days, then the central movement of chromatin would not have time to develop, because it has already been shown in the duct cells that the central migration comes after about 5 days.

For a number of years it was felt that the presence of intranuclear inclusion bodies was strong evidence for the existence of

a virus, but that point of view has lost ground by the researches of Luger and Lauda,[38] Lee,[39] Blackman,[29] and recently Olitsky and Harford.[28] These investigators report the formation of inclusions by the use of chemical agents, including metals, such as lead, iron, aluminum and arsenicals. In addition, Olitsky and Harford [40] produced inclusions by the use of virus-free brain tissue.

According to these reports from the literature the question arises, are the intranuclear inclusions accompanying cellular reactions produced in the monocytes of the guinea pig by the chemical methods of Olitsky and Harford identical with those produced by the virus? It has been shown that the cytological picture in the monocytes under the action of the virus is different from the chemical inclusions, but on the other hand the virus has produced a reaction in the nuclei of liver cells which is practically identical with the chemically produced inclusions of the monocytes. The cytology of the kidney and liver nuclei pictured by Blackman after lead poisoning are quite similar to those we find in the liver after inoculation of submaxillary gland virus, and similar to those obtained in monocytes by Olitsky and Harford, and both of these are similar to the earliest stages in nuclear reaction to a virus. A final answer cannot yet be given, but the simplest suggestion based on facts at hand is that different results are a measure of different degrees of severity of action on the cell by the agents employed, namely that the liver cell under virus action, and the monocyte under chemical action, are responding to a mild stimulus, and thus most of the cells reach only an early stage in nuclear reaction, whereas the monocytes and duct cells under virus action are severely affected and go to the full extent of the response.

Our data give us no information as to whether the intranuclear inclusions are aggregates of particles or are only cellular reaction products. Markham [6] is of the opinion from his study on monocytes in the living condition that there are present in the nucleus ". . . units comparable to the elementary bodies found in other virus infections." Our fixed and stained preparations in which the techniques employed were different from his, revealed nothing of this granular nature. The only structures suggestive of elementary bodies in our studies were observed in the cytoplasmic

inclusions of the duct cells. Their size (400 $\mu\mu$) is larger however than that given for the elementary bodies of other cytoplasmic inclusions. There are as yet no detailed studies on the size of the submaxillary virus by filtration methods. Cole and Kuttner [1] noted that it was too large to pass a Berkefeld N filter.

Viability of an Inclusion-Bearing Cell

The opinion has sometimes been expressed that a cell which bears an inclusion is dying; on the other hand, we have found records by Ludford and Findlay [41] and Goodpasture [42] that cells which contain cytoplasmic inclusions of fowl pox divide by mitosis. So far, however, there is no record of mitosis in cells that contain intranuclear inclusions, although amitotic division in these cells has been described by Lipschütz [20] in vaccinia, and by Ivanovics and Hyde [23] in tissue cultures infected with virus III. In our studies we have encountered this phenomenon of amitosis in monocytes and macrophages which contained inclusion bodies.

All of this evidence points to a certain degree of vital activity and the cells which are capable of such response are, of course, not dead. Just how soon they are going to die is difficult to determine and it is still more difficult to determine whether the presence of the inclusion body will cause them to die sooner than neighboring cells which lack inclusion bodies. In regard to the viability of the submaxillary duct cell inclusion, the evidence brought out by Pearson [14] and further advanced by Cowdry [43] that these cells represent dead cells in a state of static equilibrium, because of their resistance to peritoneal autolysis, seems to us rather unsatisfactory evidence of death since it might also represent a static condition of life of the cell making it more resistant to autolytic processes in the peritoneal cavity. Pearson [14] has suggested that the absence of mitochondria in the inclusion-bearing duct cells is evidence that they are dead. However, Sabin, Doan and Cunningham [44] observed that macrophages in tissue culture lacked mitochondria and these cells were definitely alive. In our studies we found some regions in which the state of the nuclear reaction to the inclusion had progressed quite a bit further than the usually observed "old" state, and these changes represented distinct autolytic effects (Fig. 25). These zones of autolysis were surrounded

by extensive tissue destruction and the effect may have been due to influences other than the virus; still it represents valuable evidence that the cells can undergo death changes, and when they do die the process is similar to that of other cells. This evidence would therefore place the intranuclear inclusions in the submaxillary duct cells in a "static life state" rather than one of death. The fact that these inclusions are said to persist for long periods of time, which we could verify indirectly by the fact that we never encountered "young" stages of inclusion formation, such as those of Figures 16 to 18, in any of the guinea pigs used as virus sources, brings out further the theory of viability of these cells.

Studies of Autolysis on rats show that at body temperature cells disintegrate in 18 hours after death of the animal, but the inclusion bodies in the duct cells of the guinea pig develop slowly over a period of at least 2 weeks, which fact may be additional evidence that an inclusion-bearing cell is still capable of vital activities. Scott and Pruett [45] in their cytological study of the volume of inclusion bodies present facts which may be interpreted as evidence for the idea that the inclusion-bearing cells are alive. They have given careful measurements of nucleocytoplasmic relations in salivary gland duct cells, with and without inclusion bodies. If one goes a step further with their data and calculates the nucleocytoplasmic relations after subtracting the volume of the inclusion body from the volume of the nucleus, the ratio of nucleus to cytoplasm of infected cells is approximately normal, which indicates that during the great hypertrophy of the nucleus and cytoplasm, physiological ratios characteristic of the normal living cell were maintained.

The viability of inclusion-bearing cells is still further championed by Nicolau and Kopciowska,[46] who report that such cells are not only alive but contain inclusions because they are particularly resistant to the action of the virus. Nicolau, Kopciowska and Mathis [47] go so far as to state that in mice infected with yellow fever inclusions in the brain begin to recede and disappear by the 11th to the 14th day after infection and that the same cells which carried the inclusions eliminated them from the nucleus and returned to a normal condition. We have no definite evidence, however, from our studies to suggest anything of a similar regenerative nature with the submaxillary gland virus.

Centrifugation

Evidence that centrifugation does not kill the cells but instead that tissue cells can withstand centrifugal forces sufficient to accumulate the chromatin at one pole is given by Guyer and Claus,[48] and MacDougald, Beams and King,[49] so that we may assume that the stratification of substances we observe has taken place in a living system and not in cells that were killed sometime prior to the results obtained.

The previous work with herpes inclusions [7] demonstrated that they were lighter than any normal constituents of the nucleus, but in our study on the intranuclear inclusions of duct cells the bodies proved to be heavier than the nuclear sap, but apparently lighter than the chromatin. The same result was observed in fox encephalitis. No generalization can be made until more cells affected by other viruses have been centrifuged but the differences between herpes and other viruses may prove significant in that the herpes inclusions are of the granular particulate type (type A of Cowdry [50]) while those in the monocytes and submaxillary duct cells, as well as those in the meningeal cells of fox encephalitis, under fixed and stained conditions, are a hyaline non-granular type, similar to the "chemical inclusions."

Considerable evidence is available in the work of MacCallum and Oppenheimer [51] and Schlesinger [52] on the absolute specific gravity of various viruses, but these data cannot be utilized in a study of the relation between inclusion bodies and virus until some method is devised to determine the absolute specific gravity values of chromatin, nuclear sap and other cell organelles.

Summary and Conclusions

1. A submaxillary gland virus of guinea pigs was found which proved to be more virulent than that previously reported in the literature in that it produced death regardless of the region in which the inoculation was made, whereas the submaxillary gland virus of guinea pigs usually kills only after intracerebral inoculation.

2. Young virus-free guinea pigs taken from a colony that furnished the virus were slightly more resistant to the action of the virus than guinea pigs of the same age taken from a colony in which all the members were free from the virus.

3. There is some evidence that the young guinea pigs are more resistant to the action of the virus than the full grown animals of the same stock.

4. This virus failed to maintain itself in brain to brain passages which agrees with results obtained when a less virulent type was used.

5. The symptoms and histopathology of the disease follow the same course as described by others for this virus.

6. Giant cells of both the epithelioid and foreign body types have been found in all tissues of the body where inclusion-bearing monocytes may be seen.

7. Intranuclear inclusions have been observed in lymphocytes, monocytes, macrophages, endothelial cells, cells of the tubules of the kidney, follicles of the thyroid, theca cells of the ovary, interstitial and possibly germinal cells of the testicle, epithelial cells of the epididymis, adrenal and a few nerve cells of the brain cortex.

8. Inclusion-bearing monocytes appear in numerous tissues remote from the site of injection.

9. The inclusions are frequently composed of two parts, an acidophilic portion and an acidobasophilic portion.

10. The stages of the nuclear reactions in monocytes to the developing intranuclear inclusions have been divided into young, intermediate and late. The young stages are those in which the halo is small, the chromatin discrete, and the linin network extensive with little or no margination. The intermediate stages are those in which about half of the chromatin has marginated, and the rest clumped, but still showing the particulate nature of the material forming the clumps. The late stages are those in which the chromatin forms a layer of uniform thickness. In very late stages a few granules of chromatin may return to the surface of the inclusion body.

11. The ultimate fate of the inclusion-bearing monocyte sometimes is autolysis. More frequently the monocyte is involved in the giant cell formation which in turn may undergo autolysis.

12. Intranuclear inclusions of monocytes may be found free in the tissue spaces and there phagocytozed by macrophages. This is taken as evidence that they are more resistant than the cells producing them.

13. Intranuclear inclusions develop in the duct cells of the

submaxillary gland as early as the 6th day after inoculation, and complete their development to the old stage 21 to 24 days after inoculation, which is 15 to 19 days after they first appear. Cytoplasmic inclusions develop about the middle of the cycle.

14. The early stages of nuclear reactions in the submaxillary gland duct cells include those changes that were called "early" and "intermediate" in the monocytes. The intermediate stages in duct cells involve migration of some of the granules of chromatin to the inclusion body, but these are scattered over the inclusion and are not clumped. The late stages are those in which most of the chromatin has migrated to the inclusion body and has aggregated into clumps.

15. The reaction of nuclei to inclusions of virus origin have been compared to the chemically produced inclusions of Olitsky and Harford and it is suggested that the differences are due to those of intensity of the reaction on the nucleus rather than differences in quality.

16. Several lines of evidence indicate that cells containing intranuclear inclusions are living and not dead.

17. Centrifugation of inclusion-bearing submaxillary duct cells at young and intermediate stages reveals that the chromatin is the heaviest element in the nucleus, that the inclusion is next, and that the nuclear sap is lightest, which results are contrary to those found for herpes. The cytoplasmic inclusions are as heavy as the nucleus.

REFERENCES

1. Cole, Rufus, and Kuttner, Ann G. A filterable virus present in the submaxillary glands of guinea pigs. *J. Exper. Med.*, 1926, **44**, 855–874.
2. Kuttner, Ann G. Further studies concerning the filtrable virus present in the submaxillary glands of guinea pigs. *J. Exper. Med.*, 1927, **46**, 935–956.
3. Scott, Gordon H. Studies on the submaxillary virus of guinea pigs. I. The effect of duct ligation and pilocarpine administration upon the cellular response to the virus. *J. Exper. Med.*, 1929, **49**, 229–236.
4. Andrewes, C. H. Immunity to the salivary virus of guinea-pigs studied in the living animal, and in tissue-culture. *Brit. J. Exper. Path.*, 1930, **11**, 23–34.
5. Kuttner, Ann G., and T'ung, T'sun. Further studies on the submaxillary gland viruses of rats and guinea pigs. *J. Exper. Med.*, 1935, **62**, 805–822.
6. Markham, Floyd S. A study of the submaxillary gland virus of the guinea pig. *Am. J. Path.*, 1938, **14**, 311–322.
7. Lucas, Alfred M., and Herrmann, Walter W. Effect of centrifugation on herpetic intranuclear inclusions with a note on cytoplasmic inclusions of unknown origin in the rabbit cornea. *Am. J. Path.*, 1935, **11**, 969–976.
8. Lucas, Alfred M. Ultracentrifugation of intranuclear inclusions in the submaxillary glands of guinea pigs and ground moles. *Am. J. Path.*, 1936, **12**, 933–947.
9. Cowdry, E. V. The microchemistry of nuclear inclusions in virus diseases. *Science*, 1928, **68**, 40–41.
10. Beams, J. W. An apparatus for obtaining high speeds of rotation. *Rev. Scient. Instruments*, 1930, **1**, 667–671.
11. Beams, J. W., and Pickels, E. G. The production of high rotational speeds. *Rev. Scient. Instruments*, 1935, **6**, 299–308.
12. Hudson, N. Paul, and Markham, Floyd S. Brain to brain transmission of the submaxillary gland virus in young guinea pigs. *J. Exper. Med.*, 1932, **55**, 405–415.
13. Markham, Floyd S., and Hudson, N. Paul. Susceptibility of the guinea pig fetus to the submaxillary gland virus of guinea pigs. *Am. J. Path.*, 1936, **12**, 175–182.
14. Pearson, E. F. Cytoplasmic inclusions produced by the submaxillary virus. *Am. J. Path.*, 1930, **6**, 261–274.
15. Wilson, Edmund B. The Cell in Development and Heredity. The Macmillan Company, New York, 1925, Ed. 3.
16. Doan, Charles A., Sabin, Florence R., and Forkner, Claude E. The derivation of giant cells with especial reference to those of tuberculosis. *J. Exper. Med.*, 1930, **52**, *Suppl.* 3, 89–111.

17. Forkner, Claude E. The origin and fate of two types of multinucleated giant cells in the circulating blood. *J. Exper. Med.*, 1930, **52**, 279–297.

18. Unna, P. G. Die Histopathologie der Hautkrankheiten. Lehrbuch der Speciellen Pathologischen Anatomie, Orth, J. A. Hirschwald, Berlin, 1894, **2**, 151, 156, 263, 446, 641, 646.

19. Tyzzer, E. E. The histology of the skin lesions in varicella. *J. Med. Research*, 1905–06, **14**, 361–392.

20. Lipschütz, B. Untersuchungen über die Ätiologie der Krankheiten der Herpesgruppe (Herpes zoster, Herpes genitalis, Herpes febrilis). *Arch. f. Dermat. u. Syph.*, 1921, **136**, 428–482.

21. Carrel, Alexis, and Ebeling, Albert H. The fundamental properties of the fibroblast and the macrophage. II. The macrophage. *J. Exper. Med.*, 1926, **44**, 285–305.

22. Lewis, Margaret Reed. The formation of macrophages, epithelioid cells and giant cells from leucocytes in incubated blood. *Am. J. Path.*, 1925, **1**, 91–100.

23. Ivanovics, G., and Hyde, R. R. A study of rabbit virus III in tissue culture. *Am. J. Hyg.*, 1936, **23**, 55–73.

24. Jackson, Leila. An intracellular protozoan parasite of the ducts of the salivary glands of the guinea-pig. *J. Infect. Dis.*, 1920, **26**, 347–350.

25. Goodpasture, Ernest W., and Talbot, Fritz B. Concerning the nature of "protozoan-like" cells in certain lesions of infancy. *Am. J. Dis. Child.*, 1921, **21**, 415–425.

26. Farber, Sidney, and Wolbach, S. Burt. Intranuclear and cytoplasmic inclusions ("protozoan-like bodies") in the salivary glands and other organs of infants. *Am. J. Path.*, 1932, **8**, 123–136.

27. Haagen, E. Virusmorphologie und Entstehung von Einschlusskörperchen. *Zentralbl. f. Bakt.*, Pt. 1, I. Abt. Referate, 1937, **125**, 489–496.

28. Olitsky, Peter K., and Harford, Carl G. Intranuclear inclusion bodies in the tissue reactions produced by injections of certain foreign substances. *Am. J. Path.*, 1937, **13**, 729–748.

29. Blackman, S. S., Jr. Intranuclear inclusion bodies in the kidney and liver caused by lead poisoning. *Bull. Johns Hopkins Hosp.*, 1936, **58**, 384–403.

30. Goodpasture, Ernest W. A study of rabies, with reference to a neural transmission of the virus in rabbits, and the structure and significance of Negri bodies. *Am. J. Path.*, 1925, **1**, 547–582.

31. Covell, W. P., and Danks, W. B. C. Studies on the nature of the Negri body. *Am. J. Path.*, 1932, **8**, 557–572.

32. Nicolau, S., and Kopciowska, L. Étude sur la morphogénèse des corps de Negri. *Ann. Inst. Pasteur*, 1934, **53**, 418–437.

33. Cowdry, Edmund V. The supravital staining of vaccine bodies. *J. Exper. Med.*, 1922, **36**, 667–684.

34. Taniguchi, T., Hosokawa, M., Kuga, S., and Fujino, T. A new method of staining viruses of variolo-vaccinia and varicella, and the nature of cell-inclusions in virus diseases. *Japanese J. Exper. Med.*, 1934, **12**, 91–99.

35. Paschen, E. Pocken. Handbuch der pathogenen Mikroorganismen, Kolle, W., Kraus, R., and Uhlenhuth, P., editors. Gustav Fischer, Jena, 1930, Ed. 3, **8**, Pt. 2, 821–910.

36. Goodpasture, E. W., Woodruff, Alice M., and Buddingh, G. J. Vaccinal infection of chorio-allantoic membrane of the chick embryo. *Am. J. Path.*, 1932, **8**, 271–282.

37. Burnet, F. M., and Andrewes, C. H. Ueber die Natur der filtrierbaren Vira. Ueberblick über neuere Virusuntersuchungen, unter besonderer Berücksichtigung der einschlägigen Arbeiten aus dem National Institute for Medical Research, London. *Zentralbl. f. Bakt.*, 1933, **130**, 161–183.

38. Luger, A., and Lauda, E. Über oxychromatische Veränderungen am Zellkern. (Auf Grund von Untersuchungen von Herpes simplex, Zoster, Varizellen, Variola, und Karpfenpocke.) Ein Beitrag zur Kenntnis und Wertung einschlussartiger Gebilde. *Med. Klin.*, 1926, **22**, 415–417, 456–458, 493–497.

39. Lee, Jack. Nuclear changes following intravenous injection of various solutions. *Proc. Soc. Exper. Biol. & Med.*, 1933, **31**, 383–385.

40. Olitsky, Peter K., and Harford, Carl G. Further observation on intranuclear inclusions produced by non-virus materials. *Proc. Soc. Exper. Biol. & Med.*, 1938, **38**, 92–94.

41. Ludford, R. J., and Findlay, G. M. The ultra-microscopic viruses: II. The cytology of fowl-pox. *Brit. J. Exper. Path.*, 1926, **7**, 256–264.

42. Goodpasture, Ernest W. Cellular inclusions and the etiology of virus diseases. *Arch. Path.*, 1929, **7**, 114–132.

43. Cowdry, E. V. Inapparent virus diseases. *Scient. Monthly*, 1937, **45**, 266–275.

44. Sabin, F. R., Doan, A., and Cunningham, R. S. Discrimination of two types of phagocytic cells in the connective tissues by the supravital technique. *Contrib. Embryol.*, 1925, **16**, 125–162.

45. Scott, Gordon H., and Pruett, Burchard S. Studies on the submaxillary virus of guinea pigs. II. The nuclear cell, nucleocytoplasmic and inclusion-nuclear indices of the affected cells. *Am. J. Path.*, 1930, **6**, 53–69.

46. Nicolau, S., and Kopciowska, L. La morphologie de l'inframicrobe herpétique dans le tissu des animaux infectés expérimentalement et le mécanisme de la formation des inclusions qu'il engendre dans les cellules. *Ann. Inst. Pasteur*, 1938, **60**, 401–431.

47. Nicolau, S., Kopciowska, L., and Mathis, M. Étude sur les inclusions de la fièvre jaune. *Ann. Inst. Pasteur*, 1934, **53**, 455–508.

48. Guyer, M. F., and Claus, P. E. Recovery changes in transplanted anterior pituitary cells stratified in the ultracentrifuge. *Biol. Bull.*, 1936, 71, 462–468.

49. MacDougald, T. J., Beams, H. W., and King, R. L. Growth of ultracentrifuged cells in tissue culture. *Proc. Soc. Exper. Biol. & Med.*, 1937, **37**, 234–235.

50. Cowdry, E. W. The problem of intranuclear inclusions in virus diseases. *Arch. Path.*, 1934, **18**, 527–542.

51. MacCallum, W. G., and Oppenheimer, Ella Hutzler. Differential centrifugalization — a method for the study of filtrable viruses, as applied to vaccinia. *J. A. M. A.*, 1922, **78**, 410–411.

52. Schlesinger, M. Über das spezifische Gewicht von Virus- und Bakteriophagen-elementen und seine bedeutung für die Erforschung ihrer Nature. *Biodynamica*, 1935, No. 4, 1–4.

DESCRIPTION OF PLATES

Drawings were made with a camera lucida at a magnification of × 3250 and are reduced to a magnification of × 1800. The direction of centrifugal force in Figs. 12, 13, 14, 26, 27 and 28 is indicated by an arrow beside each figure.

AIN = acidophilic inclusion; ABIN = acidobasophilic inclusion; CIN = cytoplasmic inclusion; ININ = intranuclear inclusion in cytosome of a macrophage; LUMEN = lumen of a submaxillary gland duct; and NL = nucleolus.

The cells shown in Figs. 1–15 were stained with Ehrlich's hematoxylin and eosin stain, and all except those in Figs. 9 and 10 were fixed in Zenker's fluid plus acetic acid. In these 2 cases the fixative was an alcohol and corrosive sublimate mixture.

PLATE 55

FIG. 1. An early stage in the formation of an intranuclear inclusion. A monocyte from the meninges of the brain of an adult guinea pig, which died 5 days after an intracerebral inoculation of 20 per cent submaxillary gland virus.

FIG. 2. A monocyte with two moderately large intranuclear inclusions. The nuclear reaction to the inclusion is at about the same stage as in Fig. 1. From the same slide as Fig. 1.

FIG. 3. An early stage in the nuclear reaction to an inclusion formation but later than that illustrated in Figs. 1 and 2. A monocyte from the meninges of the brain of an adult guinea pig, 1 of 3 animals in Group III, Table II, which died 8 days after intracerebral inoculation.

FIG. 4. An intermediate stage in the nuclear reaction to an inclusion formation. A monocyte from the meninges of the brain of an adult guinea pig, Group II, Table II, which was sacrificed at coma stage 4½ days after intracerebral inoculation. The acidobasophilic body forms a cap at one side of the acidophilic body.

FIG. 5. About the same stage of nuclear reaction as shown in Fig. 4. Taken from the same slide. The acidophilic and acidobasophilic bodies are separate, a condition that may be found occasionally.

FIG. 6. A late stage in nuclear reaction. Most of the chromatin, except the nucleolus, is completely marginated. Acidophilic and acidobasophilic inclusions are shown. A large monocyte taken from the same slide as that shown in Fig. 3.

FIG. 7. The latest stage of nuclear reaction observed until autolysis sets in. The marginated chromatin shows a slight tendency to aggregate into regular clumps along the margin. A small amount of chromatin has returned to the surface of the inclusion body. A large monocyte from the same slide as that shown in Figs. 4 and 5.

FIG. 8. A large monocyte which has phagocytozed three intranuclear inclusions liberated from other cells. Its own nucleus is in a late stage of nuclear reaction. A cell from the meninges of the brain of adult guinea pig No. 129, Table III, which died 7 days after intracerebral inoculation.

FIG. 9. A binucleated large monocyte. The nucleus to the left shows an early stage of reaction and the one to the right an intermediate stage. From the meninges of a 5 weeks old guinea pig (No. 149) sacrificed at coma stage 5 days after intracerebral inoculation.

FIG. 10. A small epithelioid giant cell undergoing autolysis. The inclusions are nearly obscured by the liquefied chromatin. From the same slide as Fig. 9.

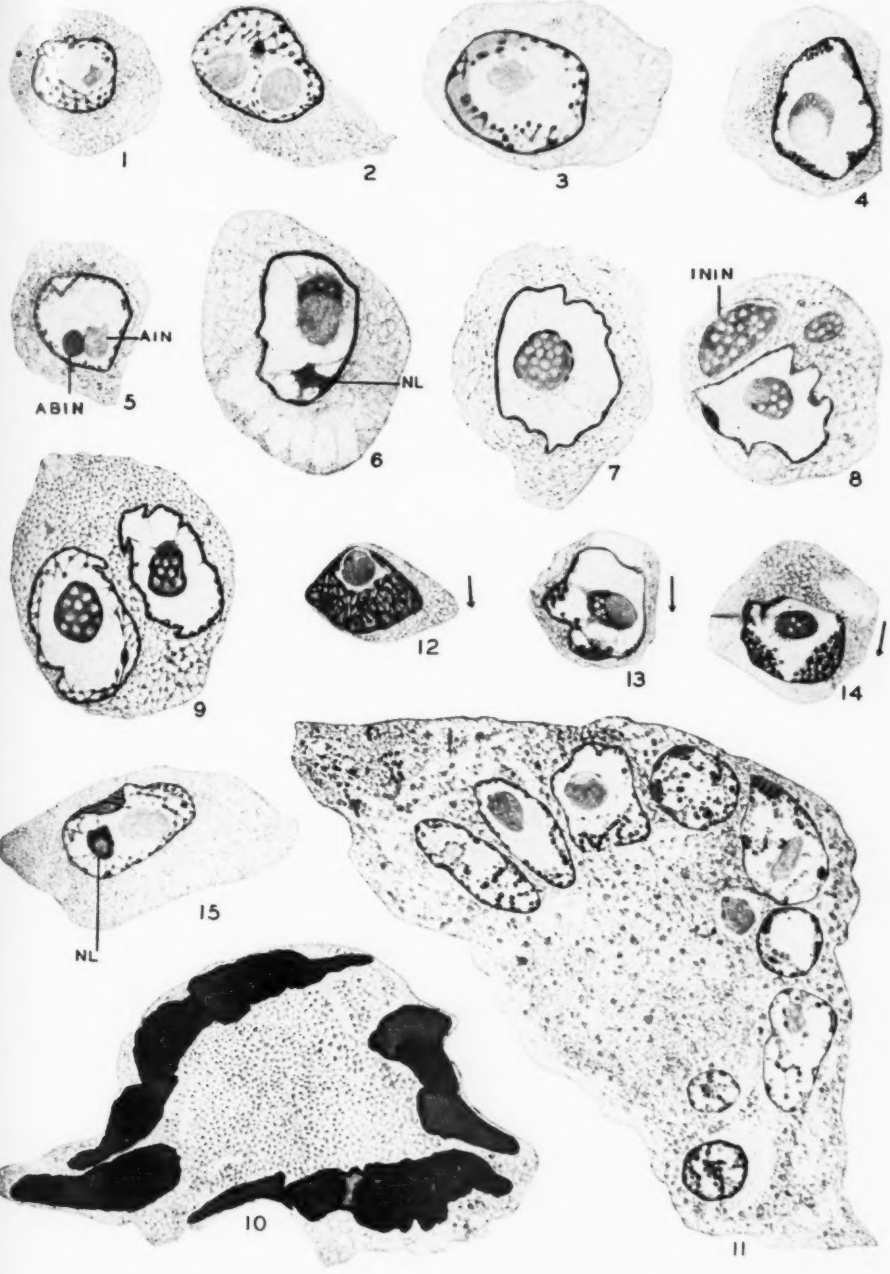
FIG. 11. An epithelioid giant cell showing varying stages of nuclear reaction to inclusion bodies. Some nuclei are undergoing autolysis or else have been phagocytozed. From the meninges of a guinea pig, Group I, Table II, which died 4 days after intracerebral inoculation.

FIG. 12. A lymphocyte with an intranuclear inclusion which has been centrifuged for 30 minutes at forces between 300,000 to 550,000 times gravity. Oxychromatin and basichromatin moved to the centrifugal pole and the inclusion body is pushed to the opposite pole. From the meninges of a guinea pig, Group I, Table II, which died 4 days after intracerebral injection.

FIG. 13. A monocyte which has been centrifuged long enough to pull much of the chromatin toward the centrifugal pole but which does not show much displacement of the inclusion body. From the same slide as Fig. 12.

FIG. 14. A monocyte which contains a greater quantity of chromatin than Fig. 13. The inclusion body is displaced slightly toward the centripedal pole. From the same slide as Fig. 12.

FIG. 15. Inclusion-bearing cell from the liver. The karyosome and plasmosome nucleoli are toward the left and the acidophilic inclusion to the right of the center. A 5 weeks old guinea pig, No. 140, Table IV, which had been injected with the virus into the spleen 7 days before death.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FIGS. 16–21 show nuclear changes in the submaxillary gland duct cells following injection of the virus. Fixation was in Zenker's fluid plus acetic acid and sections were stained with hematoxylin and eosin.

FIGS. 22–25 show submaxillary gland duct cells fixed in alcohol and corrosive sublimative and stained with Feulgen's thymonucleic acid technique with a light green counterstain in order to show the distribution of basichromatin and the development of the cytoplasmic inclusions.

FIGS. 26–28 show submaxillary gland duct cells which had been centrifuged for 1 hour at 300,000 to 550,000 times gravity. All cells were fixed in alcohol and corrosive-sublimate. The cells in Figs. 26 and 27 were stained with Ehrlich's hematoxylin and eosin, and the Feulgen reaction was done on Fig. 28.

FIG. 16. An early stage in the nuclear reaction toward the forming inclusion body. The chromatin is in the form of discrete granules. Very little hypertrophy of the cell is present. From same animal from which Fig. 15 was taken.

FIG. 17. A stage similar to Fig. 16 except that the chromatin is somewhat more clumped. From a guinea pig, Group II, Table II, which died 11 days after inoculation of the virus into the submaxillary gland.

FIG. 18. Still in the "early" stage but showing a greater degree of margination than Fig. 17; none of the chromatin, however, has returned to the inclusion body. The inclusion body is composed of two portions as in the monocytes. Definite hypertrophy of cell and its nucleus is seen. From the same slide from which Fig. 17 was taken.

FIG. 19. An "intermediate" stage in reaction of the nucleus to the inclusion body. Although the chromatin has not completely marginated some has returned to the inclusion body. The cell and its nucleus is hypertrophied. From guinea pig No. 106, Table I, which died 13 days after inoculation in the submaxillary gland region.

FIG. 20. A late "intermediate" stage. Most of the chromatin has returned to the inclusion but it has not clumped together as in the typical old stage. The cell and its nucleus are hypertrophied. From a young guinea pig, Group IV, Table II, which had been sacrificed 21 days after inoculation of virus.

FIG. 21. An "old" stage typical of the condition found in stock animals carrying the virus. Chromatin which has moved to the inclusion body is clumped. A greatly hypertrophied nucleus and cell is seen. From guinea pig No. 42, Table II.

FIG. 22. An early stage in the formation of a cytoplasmic inclusion body. The nuclear reaction is intermediate, about the same as in Fig. 19. The cytoplasmic inclusion body in this cell is on the side opposite the lumen of the duct. From guinea pig No. 105, Table I, which died 17 days after inoculation.

FIG. 23. Four cytoplasmic inclusions. The nucleus shows about the same degree of development as that shown in Fig. 20. From the same slide as Fig. 22. From this slide it was estimated that the individual granules of the cytoplasmic inclusion were about 400 millimicrons in diameter.

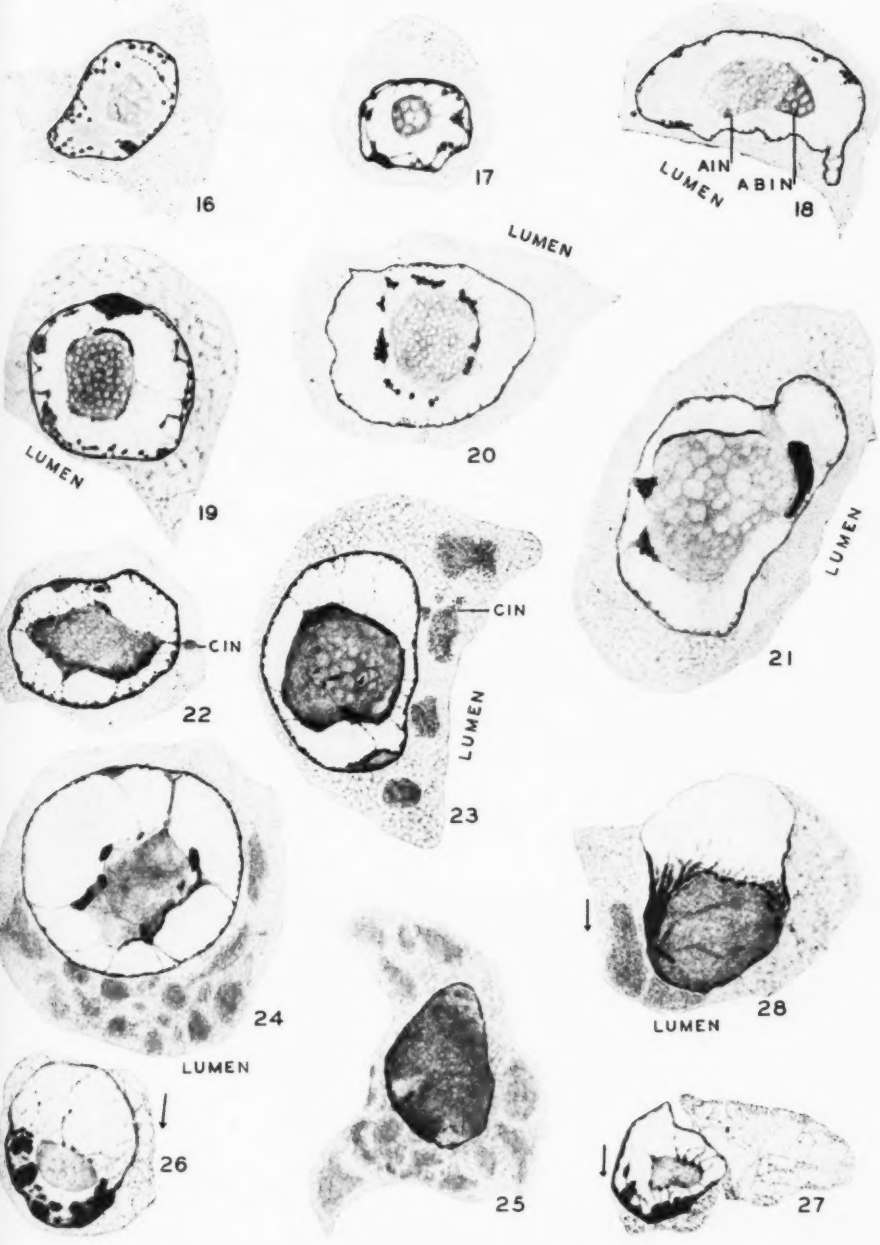
FIG. 24. Numerous cytoplasmic inclusions. The nucleus is of the old type, similar to that shown in Fig. 21. From guinea pig No. 121, Table III, which was sacrificed on the 24th day after inoculation.

FIG. 25. An inclusion-bearing cell undergoing autolysis. Liquefaction of chromatin is seen. Both nuclear and cytoplasmic inclusions are obscured. From the same slide as Fig. 24.

FIG. 26. A young stage corresponding to Figs. 16 to 18 in which little if any chromatin has migrated to the inclusion body. The oxychromatin and basichromatin are the heaviest, the inclusion intermediate, and the nuclear sap the light element in the nucleus. From guinea pig No. 117, Table I, which died 10 days after inoculation.

FIG. 27. An intermediate stage similar to Fig. 19 in which only few chromatin granules have returned to the inclusion body. These are not dislodged by centrifugation. From guinea pig No. 107, Table I, which died 14 days after inoculation.

FIG. 28. A late "intermediate" stage similar to Fig. 20. From guinea pig No. 105, Table I, which died 17 days after inoculation.



Rosenbusch and Lucas Submaxillary Gland Virus of Guinea Pig

THE HISTOLOGY OF ORBITAL AND OTHER FAT TISSUE DEPOSITS IN ANIMALS WITH EXPERIMENTALLY PRODUCED EXOPHTHALMOS *

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In the many attempts to produce exophthalmos experimentally, stimulation of the sympathetic nerves which causes a contraction of the smooth muscles of the orbit has almost invariably been used.[1-5] None of these methods has produced an exophthalmos comparable to the more marked clinical cases, although some degree of proptosis has been obtained. Recently examination of the orbital contents of animals with exophthalmos produced by injection of extract of the anterior lobe of the pituitary gland revealed many features characteristic of clinical exophthalmos following thyroidectomy (Smelser [6]). The weight of the orbital contents, muscles, lacrimal glands and fat tissue deposits was increased in these animals and the fat tissue, and to some extent the muscles, became edematous. The edema, associated with some round cell infiltration of the orbital fat tissue and muscles, presented essentially the same condition as that found in orbital tissues in exophthalmos following thyroidectomy. The proptosis produced was marked (not simply a widened palpebral fissure) and persisted postmortem. The protrusion could be convincingly demonstrated and measured by a roentgenogram of the orbit. The position of the bulb was indicated by a silver marker placed on the apex of the cornea.

These observations assume especial importance because of clinical studies of exophthalmos which have been made in recent years.[7-10] All of these studies have been concerned with malignant or progressive exophthalmos which increased following thyroidectomy. Similarly, the exophthalmos produced by injection of extract of the anterior lobe of the pituitary gland is most severe when the metabolic rate is low (Friedgood,[11] Smelser [12]), or in the absence of the thyroid (Marine and Rosen,[13] and Smelser [6, 14]). Examination of orbital contents from patients with exophthalmos

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has revealed in many instances enlarged extraocular muscles which are found on histological examination to be edematous and invaded by masses of round cells. Other cases are reported in which edema and hypertrophy of the orbital fat and connective tissue are considered to be the cause of the exophthalmos.[15–17] Further evidence indicates that the edema is general throughout the orbit, affecting both the fat tissue and the muscles.[6] Study of biopsy specimens from different regions or muscles of an orbit does not, however, show them to be necessarily equally affected, thus suggesting an explanation of the various changes observed in such cases.

The production of exophthalmos and edema of fat and connective tissue of the orbit by injection of extract of the anterior lobe of the pituitary gland raises the question whether this reaction, in widely distributed tissues, is localized in the orbit or occurs generally in the fat tissue deposits.

Experimental

Nineteen full grown guinea pigs were thyroidectomized, and following a 15 to 20 day recovery period were injected with an extract of anterior lobe of the pituitary glands of beef. The extract was prepared as described earlier [18] and was essentially a well tolerated, partially purified preparation which produced body growth of hypophysectomized rats, greatly increased weight of the thyroids and ovaries in guinea pigs and of adrenals of thyroidectomized guinea pigs. An amount of extract equivalent to 250 to 500 mg. of acetone-dried powdered anterior lobe of the pituitary gland was injected daily for various periods of time. Exophthalmos became evident in most cases within 15 to 20 days, although in a few instances 30 to 40 injections were necessary. An autopsy was performed after exophthalmos had been maintained approximately 20 days. The orbital contents were removed, weighed, and the fat tissue fixed in Bouin's fluid. All animals were exsanguinated before the tissues were removed. Fat tissue from the cervical region lying lateral to the trachea and extending posteriorly from the larynx, was also fixed. Other samples of fat tissue were taken from the axilla and the kidney. The fat tissue attached to the ovary, fallopian tube and upper portion of the uterus was taken from the females, and a specimen

surrounding the ureter near the bladder from the males. By this means fat deposits in several regions were examined and compared with orbital fat tissue from the same animal. The specimens were all fixed in Bouin's fluid, embedded in paraffin and sections cut at 6μ and stained with Masson's trichrome method and the hematoxylin-eosin stain. Sections from several regions in each specimen were used in order to study representative areas. Control tissues were obtained from 6 normal and 4 thyroidectomized uninjected guinea pigs. Nine thyroidectomized animals, 4 of which received extract of liver and 5 of which received thymus extract, provided additional tissues for comparison. The beef liver and thymus extracts were prepared by the same method used for the pituitary glands and were injected in daily amounts equivalent to 1000 mg. of the acetone-dried tissue.

Results

Sixteen of the animals injected with pituitary extract became definitely exophthalmic and were autopsied. The orbital contents were carefully examined and weighed, and were fixed for histological study. Injection of pituitary extract produced a 35 per cent increase in total weight of the retrobulbar tissues. All orbital structures were involved in this increase with the exception of the ventral lacrimal gland. The orbital fat tissue was proportionally the most affected, although the dorsal lacrimal gland, because of its size, contributed more to the total weight of the orbital contents. Swelling of the extraocular muscles was of particular interest because of the emphasis placed on them in clinical studies and because their weight was constant in the control animals. These observations have been discussed more completely elsewhere.[6] Injections of liver and thymus extracts were without effect on the weight of the orbital contents of the control animals.

The structural modifications of the dorsal lacrimal and extraocular muscles will be discussed separately. The amount of orbital fat tissue in the exophthalmic animals was not only increased but its appearance was definitely changed. Normally, and in the injected control animals, orbital fat tissue was rather opaque, but in the exophthalmic animals it appeared to be translucent and gelatinous. The amount of orbital fat tissue was nearly double that of the control animals, weighing an average of 63 mg. in the

animals injected with pituitary extract and 27 to 33 mg. in the control animals. Orbital fat tissue from normal and uninjected thyroidectomized guinea pigs weighed 31 and 33 mg., and that from control animals treated with liver and thymus extract weighed 27 and 33 mg. respectively.

Orbital Fat Tissue: The orbit of the normal guinea pig contains a small amount of fat tissue which lies directly behind the bulb, surrounding the optic nerve and filling the interstices between the extraocular muscles and, to some extent, between the lobules of the large dorsal lacrimal gland. The bulk of the fat tissue lies within the muscle cone which it fills. It is traversed by nerves, small blood vessels and the striated retractor-bulbi muscles, which are very small. The entire orbital fat content was studied and sections revealed many fat cells divided by connective tissue septums into irregular lobules. Because all autopsies were performed after the animals had been exsanguinated, no extravasation occurred to confuse the study. The collagenous connective tissue septums contained in all instances a variable but small amount of fluid. This material which stained faintly with acid dyes was found in the loose connective tissue of other fat deposits and in the orbital fat tissue of other species, including man. Injection of large amounts of extract of liver and thymus gland did not affect the orbital fat tissue of 6 of the 9 treated animals. One animal seemed to have slightly more edematous material in the connective tissue than is seen normally, and 2 animals as much as in the animals in the series treated with pituitary extract.

The orbital fat tissue of the guinea pigs treated with extract of the anterior lobe of the pituitary gland differed markedly from that of the control animals (Figs. 1 and 2). The fat cells which normally constitute the major portion of each section appeared reduced in the animals injected with pituitary extract because of an edematous infiltration which seemed in most cases to occupy nearly the entire section. The connective tissue fibers appeared to be divided and dispersed in the infiltration. The edematous fluid also penetrated between fat cells, although not in as marked a degree as between the connective tissue fibers. In many instances, however, instead of a solid mass of fat cells the edematous infiltration seemed to have scattered them so that they appeared to be floating freely in the infiltrating medium.

The edematous material stained deeply with aniline blue or eosin and did not give any staining reactions characteristic of amyloid or mucoid infiltration. Although the general appearance under low magnification was homogeneous, observation of the infiltrate with higher magnification revealed many minute acidophilic granules. Orbital fat and connective tissue, and extraocular muscles from several individuals who developed exophthalmos following thyroidectomy were found to be infiltrated with a material similar in all respects to that produced experimentally in similar tissues in the guinea pig.

The relative amounts of this infiltrating material found, expressed in arbitrary units, are shown in Table I. The valuations assigned were determined without reference to serial number of the animal and without knowledge of the treatment which had been given.

Axillary Fat Tissue: Specimens were removed from the axilla at autopsy. Nearly the entire mass of fat tissue was taken so that the sections are representative of the whole. Tissue from this region in the control animals consisted of solid masses of fat cells irregularly divided by connective tissue septums. As in the orbital fat tissue, blood vessels and nerves were found in the sections, although the axillary fat tissue was less vascular than that found in the orbit, and much less connective tissue was present. Only 7 of the 19 control animals showed small amounts of edematous material similar to that noted in the normal orbital connective tissue. Injections of thymus and liver extracts were apparently without effect on the axillary fat tissue in all instances.

Fat tissue taken from the axillary region of exophthalmic guinea pigs treated with pituitary extract did not differ from that of the control animals. About one-third of the preparations revealed a small amount of edematous material in the connective tissue which in no instance was greater than that in the animals treated with extract of liver and thymus or in those that were uninjected.

Cervical Fat Tissue: The specimens of cervical fat tissue were taken from the region lateral to the trachea, near the site of the thyroid gland, which had been removed at the beginning of the experiment. Normally, fat tissue from this region is intermediate between the axillary and orbital fat tissue in general histological

TABLE I

Relative Amount of Edematous Infiltration Found in Various Parts of the Body

Animal number	Sex	Orbital fat tissue	Axillary fat tissue	Cervical fat tissue	Renal fat tissue	Ovarian or ureteral fat tissue
		Normal Uninjected Guinea Pigs				
646	♀	+	+	+	o	o
242	♀	+	o	+++	o	o
245	♀	++	+++	++	+	o
246	♀	++	o	o	o	
247	♂	+	+++		o	
238	♂	+	++	+++	o	+
		Uninjected Thyroidectomized Guinea Pigs				
303	♀	++	o	+	o	+
304	♂	++	o	+	o	o
305	♂	++	o	++	o	
454	♀	++	o	o	o	o
		Thyroidectomized Guinea Pigs Injected with Liver Extract				
271	C.F.	++++	o	+++	o	
275	C.M.	+	o	o		
301	♀	+	o	o	o	o
302	♀	++	+	o	o	o
		Thyroidectomized Guinea Pigs Injected with Thymus Extract				
267	C.F.	++	o	+++	o	
268	♂	+++	o	o	o	
270	C.M.	++++	o	o	o	
299	♀	++	++	+++	o	
300	♀	+	+	+++	o	
		Thyroidectomized Guinea Pigs Treated with Extract of the Anterior Lobe of the Pituitary Gland				
199	♀	++++	+	+++	+	++
219	♀	++++	+	+++	o	+++
225	♀	++++	o	+	o	o
241	♀	++	o	o	o	o
261	♀	++++	++	+	o	o
262	C.F.	++++	o	o	+	
263	C.F.	+++	o	o	o	
265	♀	++	o	+	o	o
273	♀	++++	+	+	o	+++
285	♀	++		+		
287	♀	++++	++	+++	++	++++
288	♀	++++	o	++	o	o
289	♀	+++		++		
290	♀	++		+		
291	♀	++++	+	+	o	+++
292	♀	++++	o	o		
294	♀	++++	o	++	++	++++
295	♂	++++	o	++	o	++
297	♂	++++	++	++	o	+++

o = A minute trace or no infiltration. + = A little infiltration observed.
++ and +++ = Intermediate degrees of infiltration.
++++ = Extreme infiltration, the entire section being affected.
All valuations were assigned without reference to serial numbers or treatment.
C.F. = Castrated Female; C.M. = Castrated Male.

appearance. There is more connective tissue and vascularity present than is seen in the specimens from the axillary region but less than in those from the orbital area. The edematous material noted in the connective tissue of the axillary and orbital fat tissue was found in this series in 11 of the 18 guinea pigs studied. The amount was greater than that found in the axillary region and nearly as much as that found in the orbit. Injection of thymus and liver extracts did not affect its distribution or amount.

Injection of extract of the anterior lobe of the pituitary had apparently little or no effect on the cervical fat tissue. Fifteen out of 19 animals exhibited a small amount of edema in the connective tissue bands, a slightly higher proportion than in the control animals, but in none of them was it more extensive than is seen normally (Figs. 3 and 4).

Renal Fat Tissue: The mass of fat tissue partly surrounding and covering the peritoneal side of the kidney was the fourth fat deposit studied. Structurally it differed considerably from all the others. No nerves and very few blood vessels passed through it and the amount of connective tissue present was the least in all the specimens studied. Sections from the normal control animals revealed a solid mass of fat cells which were not divided into irregular lobules by connective tissue septums, as were those from the cervical and orbital regions. In only one instance did the normal and thyroidectomized control animals reveal any of the edematous material characteristic of normal tissues from the orbit, neck and axilla. Injections of thymus and liver extract did not modify this condition in any instance.

The renal fat tissue from the exophthalmic animals treated with pituitary extract, however, was slightly affected. One-fourth of the specimens showed some accumulation of edematous infiltration in the connective tissue. In one instance this was sufficient to penetrate and partially disperse some of the fat cells. In no instance, however, was the condition extreme, but the deviation in these animals from that seen in the highly consistent control animals was convincing.

Ovarian Fat Tissue: The sections containing ovarian fat tissue differed from the other preparations because of the several different types of tissue present (Figs. 5 and 6). Each section passed through the ovary, fallopian tube and the uterus, and the mesen-

tery and fat tissue attached to them. The fat tissue was divided into lobules by septums and penetrated the connective tissue support of the fallopian tubes. Many smooth muscle fibers were present in this region. The edematous material noted in other normal fat tissue was present, in a very small amount, in only one specimen, and the ovarian fat tissue from the animals treated with liver extract revealed none of this infiltration. Fat tissue from this region of the guinea pigs treated with thymus extract was not studied.

Ovarian fat tissue from 10 animals treated with pituitary extract was studied. Six of these specimens contained considerable amounts of edematous material, all of them more than that in any control animal, and four contained large amounts of edematous material. The condition of 2 animals was judged as extreme as evidenced by the appearance of the orbital fat tissue.

Ureteral Fat Tissue: Very few specimens of fat tissue from the ureters of male animals were examined. Normally they consisted of irregular lobules of fat tissue separated by septums of connective tissue fibers. Some nerves and a few blood vessels penetrated this mass. The connective tissue from 1 of the uninjected animals contained a small amount of edema, such as was noted in other control animals, and a second specimen contained none (Fig. 7). No material was studied from animals injected with liver or thymus extract.

Two specimens of fat tissue were taken from the ureters of male animals treated with extract of the anterior pituitary gland. Both specimens showed considerable edematous infiltration of the connective tissue. This edematous material penetrated through the connective tissue septums and to lesser degree throughout the fat cells, tending to isolate them (Fig. 8). The infiltrating material, although much less in amount than that in the orbit, possessed the same staining characteristics and texture as the orbital and the other fat tissues.

Discussion

The data shown in Table I demonstrate that the amount of edematous material found in the fat tissue studied not only varies widely in amount between the exophthalmic and the control series of animals, but also between the various fat deposits in the same

animals. If averages of the ratings for the control animals and those treated with pituitary extract are studied, this is very apparent (Table II). Normally the orbital fat tissue contains more edematous material than any of the other tissues, axillary fat tissue very little, and renal fat tissue none at all. In the exophthalmic animals, or those injected with pituitary extract, this relationship is preserved, the orbital fat tissue containing much more edematous infiltration.

TABLE II

Average Degree of Edematous Infiltration of the Fat Deposits in Control and Exophthalmic Animals

	Orbital fat tissue	Axillary fat tissue	Cervical fat tissue	Renal fat tissue	Ovarian fat tissue	Ureteral fat tissue
Controls	1.9	0.6	1.38	0.05	0.1	0.5
Exophthalmic	3.5	0.6	1.36	0.4	1.9	2.5

The figures are the average of + signs in their respective groups. Ureteral fat tissue values represent only 4 cases.

Injection of pituitary extract increases not only the degree of edema but also the percentage of instances in which it is found (Table III). It may be worthy of note that, with the exception of the orbital, only fat tissue in the peritoneal cavity was much affected by the injection of pituitary extract.

TABLE III

Percentage of Specimens in which any Trace of Edematous Infiltration Was Found

	Orbital fat tissue	Axillary fat tissue	Cervical fat tissue	Renal fat tissue	Ovarian fat tissue	Ureteral fat tissue *
	%	%	%	%	%	%
Controls	100	36.8	61.1	0.05	14	50
Exophthalmic	100	43.7	79	26.6	60	100

* The figures on ureteral fat tissue are based on only 2 cases in each series of animals.

The difference between the various fat tissues in their reaction to pituitary extract may be, in part, explained on a basis of structure. Orbital fat tissue, which exhibits the most extreme degree of edema in exophthalmos and which even shows traces of a similar material normally, contains many septums of loose connective tissue. It is in these bands of collagenous fibers that the edematous infiltration is first found and is most extensive. Renal fat tissue, which contains practically no connective tissue, is the least affected.

This structural feature may explain why endocrine products circulating generally seem to have a rather specific effect (edema of the orbital fat and connective tissue) on a widely distributed tissue. The present study indicates that the same process as that taking place in the orbit is occurring generally in peritoneal fat deposits. It is more extreme in the orbital region because of the structure of its fat tissue. Any swelling of the orbital fat tissue, particularly in man, where it fills most of the retrobulbar space, is immediately noticeable because of its effect on the protrusion of the eye, whereas a slighter degree of edema elsewhere would pass unnoticed. The degree of edema produced in fat tissue from the peritoneal cavity was too slight, relative to the weight of the entire mass, to affect the weight or appearance except microscopically.

By histological study one can determine fairly accurately the location of the edematous infiltration in question but accurate quantitative data on the amount of water, fat and connective tissue cannot be ascertained. This can be done only by chemical analysis of the tissues in question. Such a study in its preliminary stages confirms the general observations reported here.

Conclusions

1. The injection of extract of the anterior lobe of the pituitary gland produces exophthalmos and marked edema of the orbital fat and connective tissue.

2. Fat tissues in various parts of the body are also affected by the injections of anterior pituitary extract but to a lesser degree than that in the orbit.

3. The difference in degree of edema produced in the orbit and that found in other fat tissue deposits may be due to the difference in structure of the orbital fat tissue.

4. The edematous material found in the orbit appears to be identical in composition with that found elsewhere in the same animal.

REFERENCES

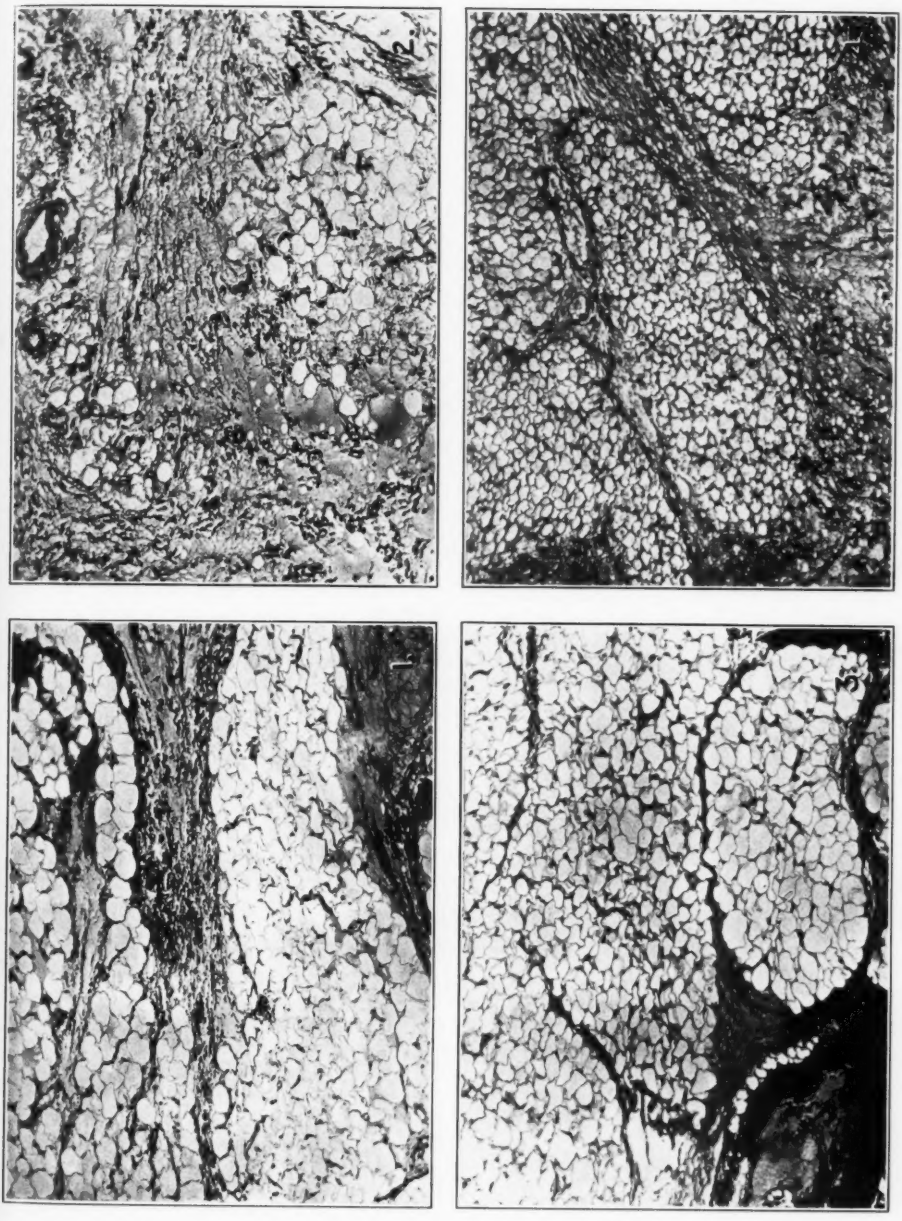
1. MacCallum, W. G., and Cornell, W. B. On the mechanism of exophthalmos. *Med. News*, 1904, **85**, 732–736.
2. Cannon, W. B., Binger, C. A. L., and Fitz, R. Experimental hyperthyroidism. *Am. J. Physiol.*, 1915, **36**, 363–364.
3. Loeb, Leo, and Friedman, Hilda. Exophthalmos produced by injections of acid extract of anterior pituitary gland of cattle. *Proc. Soc. Exper. Biol. & Med.*, 1932, **29**, 648–650.
4. Code, Charles F., and Essex, Hiram E. The mechanism of experimental exophthalmos. *Am. J. Ophth.*, 1935, **18**, 1123–1128.
5. Marine, David, Rosen, S. H., and Cipra, Anna. Further studies on the exophthalmos in rabbits produced by methyl cyanide. *Proc. Soc. Exper. Biol. & Med.*, 1933, **30**, 649–651.
6. Smelser, George K. A comparative study of experimental and clinical exophthalmos. *Am. J. Ophth.*, 1937, **20**, 1189–1203.
7. Burch, Frank E. The exophthalmos of Graves' disease. *Minnesota Med.*, 1929, **12**, 668–675.
8. Naffziger, Howard C. Pathologic changes in the orbit in progressive exophthalmos with special reference to alterations in the extra-ocular muscles and the optic disks. *Arch. Ophth.*, 1933, **9**, 1–12.
9. Wheeler, John M. Principles of modern surgery in ophthalmology. *Am. J. Ophth.*, 1934, **17**, 683–688.
10. Thomas, Henry M., and Woods, Alan C. Progressive exophthalmos following thyroidectomy. *Bull. Johns Hopkins Hosp.*, 1936, **59**, 99–113.
11. Friedgood, Harry B. Experimental exophthalmos and hyperthyroidism in guinea pigs. *Bull. Johns Hopkins Hosp.*, 1934, **54**, 48–67.
12. Smelser, George K. Treatment of experimentally produced exophthalmos with thyroxin and other iodine compounds. *Am. J. Ophth.*, 1938, **21**, 1208–1218.
13. Marine, David, and Rosen, S. H. The exophthalmos of Graves' disease; its experimental production and significance. *Am. J. M. Sc.*, 1934, **188**, 565–571.
14. Smelser, George K. Experimental production of exophthalmos resembling that found in Graves disease. *Proc. Soc. Exper. Biol. & Med.*, 1936, **35**, 128–130.
15. Moore, R. Foster. Exophthalmos and limitation of the eye movements of Graves's disease. *Lancet*, 1920, **2**, 701.
16. Moore, R. Foster. IV. Discussion: the diagnostic significance of proptosis. *Tr. Ophth. Soc. U. Kingdom*, 1923, **43**, 215–216.
17. Thomson, Edgar S. Orbital edema in exophthalmic goitre. *Am. J. Ophth.*, 1924, **7**, 27–35.
18. Smelser, George K. Chick thyroid responses as a basis for thyrotropic hormone assay. *Endocrinology*, 1938, **23**, 429–438.

DESCRIPTION OF PLATES

PLATE 57

All microphotographs were taken at a magnification of × 45.

1. Orbital fat tissue from a guinea pig (No. 275) injected with liver extract.
2. Orbital fat tissue from an exophthalmic guinea pig (No. 261) injected with pituitary extract. Note the marked edematous infiltration which disperses the connective tissue fibers and almost entirely replaces the fat cells.
3. Cervical fat tissue from an untreated thyroidectomized guinea pig (No. 305).
4. Cervical fat tissue from an exophthalmic guinea pig (No. 287). This particular specimen shows slightly more edematous infiltration than the average.



Smelser

Fat Tissue in Experimental Exophthalmos

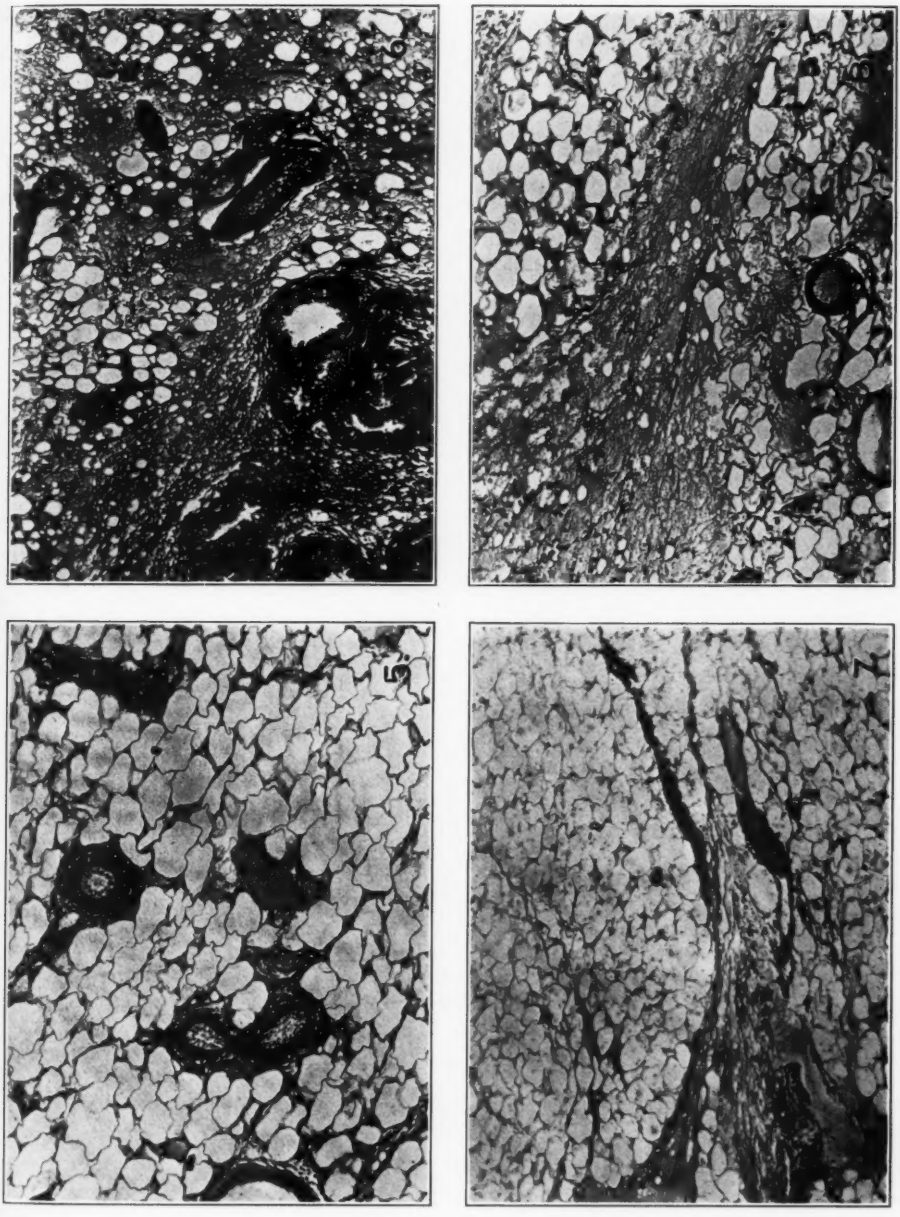
Plate 58

5. Ovarian fat tissue from an untreated thyroidectomized guinea pig (No. 303).

6. Ovarian fat tissue from an exophthalmic guinea pig (No. 294). Note the marked edema in the connective tissue and between the fat cells. Sections of the fallopian tubes are seen.

7. Fat tissue from the ureter of a normal male guinea pig (No. 238).

8. Fat tissue from the ureter of an exophthalmic male guinea pig (No. 297) injected with pituitary extract. Compare with Figs. 2 and 6.



Smelser

Fat Tissue in Experimental Exophthalmos

CEREBRAL VASCULAR DISEASE ACCOMPANYING SICKLE CELL ANEMIA *

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Patients with sickle cell anemia accompanied by cerebral vascular accidents have been observed by several authors.

Cook [1] first reported a case of a negro boy aged 7 years who expired following sudden loss of consciousness. The autopsy showed subarachnoid hemorrhage associated with cerebral softening.

Arena [2] reported the case of a negro boy aged 6 years who had headache, vertigo, stupor and right hemiplegia. A footnote to this article cited a similar case observed in a negress aged 4 years. Arena mentioned personal communications from Sydenstricker and Cooley citing 3 other cases and suggested thrombosis of cerebral vessels as a possible cause of the neurological changes.

Yater and Hansmann [3] reported a case of a negress aged 38 years who had neurological manifestations. An autopsy showed recent diapedesis of red cells into the meninges over the right hemisphere, a small area of softening 1 cm. in diameter in the cortex of the left parietal region, and a uniformly shrunken, sclerosed choroid plexus with calcium encrustations.

Ford [4] mentioned several negro children with dural sinus thrombosis who had had sickle cell anemia.

A case of a negro boy, aged 10 years, who had repeated symptoms referable to the central nervous system with residual spasticity on the right side, was reported by Kampmeier.[5]

Harden [6] described vascular changes in the retina and extremely tortuous and thickened superficial temporal vessels in 2 negro children with sickle cell anemia.

Arena [7] has also observed 4 negro boys, aged 4, 5, 6 and 10 years, who showed neurological symptoms and signs and who had sickle cell anemia.

In none of the cases thus far reported has an adequate description of the actual condition of the cerebral vessels been recorded,

* Received for publication January 19, 1939.

and no satisfactory account of the origin and nature of the cerebral clinical disturbances has been offered.

The following account of the clinical and pathological study of 2 cases of sickle cell anemia accompanied by cerebral vascular manifestations is recorded because of its contribution to the pathogenesis of the disorders of the central nervous system in sickle cell anemia and to the general problem of the pathology of the disease.

Case Reports

Case 1. *Clinical History:* A negress, aged 4 years, first entered the Duke Hospital on Feb. 8, 1934, with a history of paralysis of 3 weeks duration. At the onset of the attack the child suddenly became speechless and saliva drooled from her mouth, which drooped. Two weeks later the child lost the use of her right arm and leg. The family history was interesting in that 3 other siblings died with a similar condition. In 1 of these, sickle cell anemia had been diagnosed in our clinic. This sibling died at home after having been followed in the Duke Hospital Clinic.

Physical Examination: The temperature was 37.5° C., pulse 100, respirations 26, and the blood pressure 105/65 mm. Hg. The child could neither walk nor speak, but could comprehend commands and questions. The tongue could not be protruded or moved from side to side. The spleen was palpable 2 to 3 fingerbreadths below the costal margin. Paralysis, weakness, and spasticity of the right arm and leg were present. The reflexes were hyperactive throughout but more marked on the right side. The Babinski, Kernig and Brudzinski signs were all absent. A lumbar puncture showed no abnormality of the spinal fluid.

Accessory Clinical Findings: A fresh blood smear showed 100 per cent sickling in 4 hours; the hemoglobin was 52 per cent (7.8 gm.) Sahli, and the white blood cells numbered 24,000. The indirect van den Bergh test was slightly positive (trace). The patient was discharged from the hospital with a diagnosis of sickle cell anemia with cerebral thrombosis in the region of the left internal capsule.

In an interval of 3½ years, during which time the patient was followed in the Duke Pediatric Clinic, the use of the right arm and leg returned and speech returned almost to normal. The finer movements with the right hand, however, such as writing, buttoning clothes or holding table silver, could not be performed. A residual limp and dragging of the right leg persisted.

The patient was again admitted to the hospital 3½ years later. Fifteen hours previous to the second admission she suddenly covered the frontal region of her head with her left hand and cried out that her head hurt. She immediately crumpled to the floor, respirations became labored, and both legs became paralyzed. Inability to speak followed.

Physical Examination: The temperature was 39.0° C., pulse 120, respirations 38, and the blood pressure 150/100 mm. Hg. The child, now 7½ years of age, was small and undernourished and lay unconscious in bed with mouth and eyes open, breathing rapidly and irregularly. The lungs were clear. A soft, blowing systolic murmur was heard over the entire precordium but best over the mitral area. The eyes moved aimlessly at times but tended toward

left horizontal deviation. The spleen was not palpable. Both arms were spastic, the right more so than the left. The left leg was flaccid. Supraorbital pressure caused purposeful movements with both arms, in an attempt to knock away the examiner's hands, and the left leg became spastic. The tendon reflexes were hyperactive on both sides, more marked on the right. The abdominal reflexes were absent. The Kernig and the Babinski tests were positive bilaterally. A response to painful stimuli was present over the entire body.

Accessory Clinical Findings: The hemoglobin was 40 per cent (6.7 gm.) Sahli, red blood cells 1,500,000, white blood cells 50,000, with polymorphonuclear leukocytes 94 per cent. Sickling was 25 per cent, immediate; 95 per cent in 24 hours. The urine contained albumin (4+) and 5 to 10 granular and cellular casts per high power field. The spinal fluid showed an initial pressure of 270 mm., was bloody, with a slight xanthochromia and contained 30,000 red blood cells. The Wassermann and colloidal mastic tests were negative.

The patient was restrained, placed in a dark room, and was given sedation with an infusion of 2.5 per cent glucose. At this time the temperature was 105.8° F. No change followed and she expired quietly 15 hours later.

Autopsy Report

Macroscopic Examination: The heart was slightly dilated but not hypertrophied. The spleen (Fig. 5*a* and *b*) weighed only 1.5 gm. and measured 3.2 by 1.5 by 1 cm. It was triangular, covered with many adhesions, firm, wrinkled and grayish pink with a few small bluish areas scattered over the surface. The cut surface showed a thick capsule with prominent trabeculae and gray pulp with a few brown spots throughout. In one area a large reddish brown hemorrhage 1 cm. in diameter was present.

When the dura was opened a large amount of fluid blood was evacuated. Both subdural and subarachnoid hemorrhages in the form of blood clots covered the brain (Fig. 8), the clots being more marked over the right optic tract, anterior portion of the cerebellum, right Sylvian fissure and right insula. An area of encephalomalacia involved the left Rolandic fissure, the left posterior end of the Sylvian fissure, the left superior temporal gyrus and the entire left frontal lobe. The gyri of the left frontal and occipital lobes appeared small in contrast with those of the right except for the right middle frontal convolutions which also appeared atrophic. The right middle cerebral artery was buried in a large fresh blood clot between the right frontal and right temporal lobes. A blood clot measuring 4 cm. in diameter (Fig. 9) was found in the region of the right insula at the level of the anterior commissure.

Sections of the medulla showed a marked difference in the size of the right and left pyramidal tracts (Fig. 6), the right tract being much smaller than the left. The left lateral ventricle was greatly dilated (Fig. 9), being two to three times the size of the right.

The diameter of the middle cerebral arteries was smaller where they branched from the internal carotids, at the circle of Willis, than in the distal portions.

Microscopic Examination: Many sickled erythrocytes were seen in a preparation of heart's blood in normal saline at the time of autopsy.

The splenic structures were distorted, with a predominance of connective tissue, especially at the periphery where the capsule was wrinkled and provided with many hyaline infoldings (Fig. 1). The cells of the pulp consisted mainly of irregularly shaped lymphocytes, plasma cells and sickle shaped erythrocytes. Throughout the connective tissue there could be seen large deposits of iron and calcium, some being in the form of rings (Fig. 1), the latter under oil immersion appearing irregular and fragmented. The splenic vessels (Fig. 2) showed intimal proliferation, disrupted internal elastic lamina with iron and calcium deposits, fragmented muscular coats with pyknotic nuclei, hyaline walls with necrotic areas, and a few perivascular hemorrhages. One large hemorrhage contained pale staining sickle shaped erythrocytes, fibroblasts and minute deposits of iron pigment. The lumens of many vessels were nearly obliterated by periarterial fibrosis and intimal proliferation. Typical malpighian corpuscles were not noted.

The cerebral vessels showed endarteritis and, in several instances (Figs. 3 and 4), obliteration of the lumen with endothelial proliferation, fibroblastic reaction and hyalinization, occasional loss of internal elastic lamina, and fragmentation of the persistent internal elastic lamina with iron deposits. A few inflammatory cells were seen in the intima, media and adventitia. Perivascular hemorrhages and necrosis of the muscle layers with pyknosis and fragmentation were also present. No cholesterol crystals were noted and the elastic lamina showed thickening but no splitting, as described by Tuthill.[8] The lumens of the right middle cerebral artery and left ascending branch of the left middle cerebral artery were completely obliterated; other vessels with partial oblitera-

tion were the left middle cerebral, and right and left anterior cerebral arteries. The left internal carotid artery showed only breaks in the internal elastic lamina but no intimal thickening.

The brain contained many areas of focal necrosis usually associated with perivascular hemorrhages. Almost complete degeneration was found throughout the left frontal cortex and left pyramidal tract above the decussation. No proliferation of endothelium was noted in the minute vessels of the brain substance. The large blood clot in the region of the right insula was carefully dissected for possible aneurysm but none was found. Serial sections of the small vessels showed only complete breaks in the walls without inflammatory reaction or necrosis.

No abnormality of the aorta was noted, nor had any been noted grossly.

Capillary engorgement with sickled erythrocytes was a prominent feature in the organs studied.

Anatomical Diagnoses: History of sickle cell anemia and two cerebral accidents. Sickle cell anemia with calcification and iron pigmentation of blood vessels; extensive endarteritis obliterans of splenic and cerebral arteries; siderofibrosis of spleen; anemic infarct of left cerebrum; subarachnoid and subdural hemorrhage; and congestion of lungs with pleural adhesions.

CASE 2. *Clinical History:* A 62 year old negress was admitted to the Duke Hospital on August 10, 1936. She had been married 30 years and had had 5 pregnancies with 5 healthy children. The patient had had severe parietal headaches at fortnightly intervals. In her early forties she was committed to an institution for a year or more because of some mental aberration. Her recovery apparently had been complete.

About 3 or 4 days prior to admission to the hospital she did not appear well although there was no specific complaint. Two days prior to admission she suffered severe pain in the lower back, abdomen and legs. She consulted her physician who found her temperature to be 100° F., the blood pressure 94/60 mm. Hg., and the pulse quite rapid. "Drops" were prescribed for the heart condition and another medicine of unknown composition was also prescribed. The patient complained less of pain and, at the same time, grew less communicative. During the following 12 hours she became unresponsive and was admitted to the hospital.

Physical Examination: The temperature was 38.8° C., pulse 108, respirations 24, and the blood pressure 80/60 mm. Hg. The patient was poorly developed and undernourished, and lay quietly in bed except for purposeless movements of the extremities. The chest was slightly increased in the anterior-posterior diameter. The lungs showed hyperresonance to percussion and the breath sounds were distant. Atelectatic râles were heard. The heart was

not enlarged and the spleen was not palpable. Moderate thickening of the peripheral vessels was noted. Neurological examination showed definite lower left facial weakness and hyperactive tendon reflexes. The abdominal reflexes were absent and a questionably positive Babinski's sign was present on the left side.

Accessory Clinical Findings: The hemoglobin was 58 per cent (9 gm.) Sahli, red blood cells 3,000,000, white blood cells 10,600 with 89 per cent polymorphonuclear leukocytes. The Wassermann and Kahn tests were negative. The plasma non-protein nitrogen was 61 mg. per cent. The urine showed a slight trace of albumin and gave a positive benzidine test. A lumbar puncture disclosed normal dynamics and clear spinal fluid which was negative for blood and globulin. A spinal fluid Wassermann test was negative. Roentgenogram of the chest showed the lungs to be clear. One sickle cell preparation was reported to be negative.

The patient became stuporous and finally comatose. Fever, pulse and respirations increased and incontinence of urine and feces developed. A white blood cell count at this time was 42,000. Treatment was mainly supportive. The patient expired quietly on the 3rd hospital day.

Autopsy Report

Macroscopic Examination: The spleen weighed 250 gm., and measured 14 by 10 by 5 cm. It was covered with many adhesions and on section the pulp was red and extensive areas of infarction were present. One area of necrosis extended from the capsule through the entire diameter of the organ. The large blood vessels at the hilum showed no thrombi.

Minute hemorrhages were present in the subendocardium of the heart. Chronic passive congestion with central atrophy was noted in the liver. The aorta showed slight thickening of the intima.

The brain contained numerous minute hemorrhages (Fig. 7) from 1 to 2 mm. in diameter throughout both white and gray matter. Many softened areas were present about the hemorrhages. The blood vessels showed no abnormalities.

Microscopic Examination: Sickling of the erythrocytes was noted in a preparation made from freshly scraped splenic pulp.

The brain showed many large areas of focal necrosis, some of which were apparently unrelated to hemorrhage. Associated with the numerous hemorrhages in the brain substance were peculiar hyaline bodies resembling masses of fluid and blood cells within greatly congested capillaries. These bodies appeared somewhat like cells that had undergone extensive hyalinization. Similar hyaline bodies were seen in the infarcted areas of the splenic tissue.

The organs in general showed capillary engorgement with sickled erythrocytes.

Anatomical Diagnoses: Sickle cell anemia; hemorrhagic splenic infarct; extensive focal hemorrhages in the lungs and heart, and especially the brain; lobular pneumonia; uterine polyp; and old fibrous pleural adhesions.

Discussion

Obliterative vascular changes and thrombosis in the spleen are definitely known to be a part of the pathological changes noted in sickle cell anemia. This has been described particularly in the vessels of the spleen by Diggs.[9]

In our 1st case the alterations of the cerebral and splenic blood vessels are similar. The blood vessels in the brain and spleen show perivascular hemorrhages, hyalinization of the media, and proliferation of the intima with occasional complete obliteration of the lumen and disruption of the internal elastic lamina, the latter containing iron and calcium deposits. This suggests the possibility that the cerebral and splenic vessels are injured by the same etiological agent.

The changes in the cerebral vessels in our 1st case were similar to those described by Winkelman and Eckel[10] in infectious diseases of infancy and childhood in that there were present subintimal proliferation with thickening and secondary degeneration of the intima, disruption of the elastic lamina, mild inflammatory reaction in the media and gradual disappearance of the muscle fibers with replacement by connective tissue. In addition, deposits of iron and calcium were found in the internal elastic lamina.

The splenic changes represent those found in siderofibrosis of the spleen which has been described and discussed by Diggs.[9] He states that the size of the spleen decreases, depending on the diminished vascularity of the organ, organization of hemorrhages and infarctions, and the replacement of the residual pulp by fibrous tissue.

In studying 3 spleens from cases of sickle cell anemia, frequent encrustations of iron and calcium in the form of rings throughout the fibrosed splenic pulp have been noted. This seems very likely to be the end result of fibrosis and degeneration of splenic vessels.

The sickled erythrocytes were easily demonstrated in unfixed

scrapings and in both Zenker and formalin-fixed tissues. The most characteristic sickled cells were narrow pointed rods and elliptical shaped cells, as mentioned by Diggs and Ching.[11] In studying the organs removed from negroes who had no history of symptoms of sickle cell anemia such pointed or elliptical shaped erythrocytes were not found in either Zenker or formalin-fixed tissues. There was an alteration in the shape of the erythrocytes, however, which consisted of decrease in size and some change in shape, oat or crescent, of the cells. No pointed rods were found.

Summary and Conclusions

1. A study of 2 cases of sickle cell anemia (1 in an adult and 1 in a child) has shown that the disorder may first become manifest through the appearance of signs and symptoms indicative of cerebral vascular disease. The clinical features in such cases lead to the diagnosis of either cerebral vascular thrombosis or intracranial hemorrhages.

2. The pathological changes seen in 1 of the cases establish the fact that in sickle cell anemia the large subarachnoid cerebral arteries may undergo gradual obliteration with final complete closure through the operation of a process identical with that which results in occlusion of the splenic arteries. This process is one of endarterial intimal proliferation and not of thrombosis.

3. In a 2nd case the autopsy showed that another vascular process, quite different from endarterial intimal proliferation, also occurs in sickle cell anemia. This process develops in connection with the small intracerebral vessels and may result in multiple focal necroses and hemorrhages in the brain, in contrast with the large infarcts that characterize the proliferative obstructive process in the larger arteries. The nature of this second process is not clear.

REFERENCES

1. Cook, William C. A case of sickle cell anemia with associated subarachnoid hemorrhage. *J. Med.*, 1930, 11, 541–542.
2. Arena, Jay M. Vascular accident and hemiplegia in a patient with sickle cell anemia. *Am. J. Dis. Child.*, 1935, **49**, 722–723.
3. Yater, Wallace M., and Hansmann, George H. Sickle-cell anemia: a new cause of cor pulmonale. *Am. J. M. Sc.*, 1937, **191**, 474–484.
4. Ford, Frank R. Diseases of the Nervous System in Infancy, Childhood and Adolescence. Charles C. Thomas, Springfield, Ill., 1937, 642.
5. Kampmeier, R. H. Sickle cell anemia as a cause of cerebral vascular disease. *Arch. Neurol. & Psychiat.*, 1936, **36**, 1323–1329.
6. Harden, A. S. Sickle cell anemia — changes in the vessels and in the bones. *Am. J. Dis. Child.*, 1937, **54**, 1045–1051.
7. Arena, J. M. Cerebral vascular lesions accompanying sickle cell anemia. *J. Pediat.*, (in press).
8. Tuthill, C. R. The elastic layer in the cerebral vessels: studies of the new-born and of children. *Arch. Neurol. & Psychiat.*, 1931, **26**, 268–278.
9. Diggs, L. W. Siderofibrosis of the spleen in sickle-cell anemia. *J. A. M. A.*, 1935, **104**, 538–541.
10. Winkelman, N. W., and Eckel, John L. Arterial changes in the brain in childhood. *Am. J. Syph. & Neurol.*, 1935, **19**, 223–237.
11. Diggs, L. W., and Ching, R. E. Pathology of sickle-cell anemia. *South. M. J.*, 1934, **27**, 839–845.

DESCRIPTION OF PLATES

PLATE 59

FIG. 1. Microphotograph of spleen showing encrustations of iron and calcium, the former being in the form of rings, with diminished splenic pulp and increased connective tissue.

FIG. 2. A splenic vessel showing fragmented elastic lamina, proliferation of the intima and perivascular hemorrhage.

FIG. 3. Cross section of left middle cerebral artery showing partial occlusion and fragmented elastic lamina.

FIG. 4. High power of inset (Fig. 3) showing fragmented internal elastic lamina with iron encrustations.

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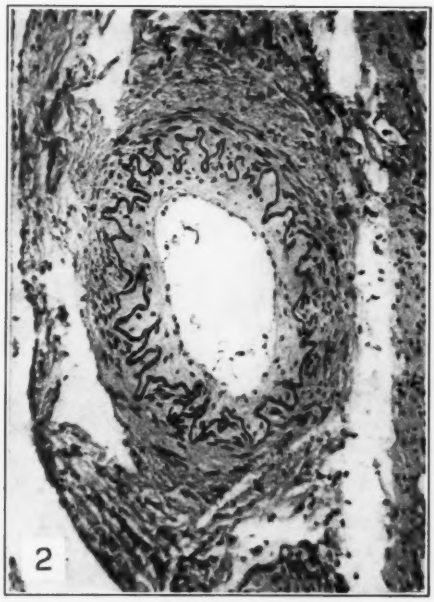
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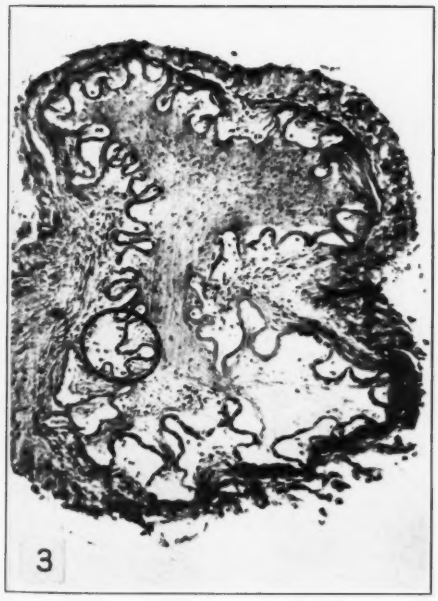
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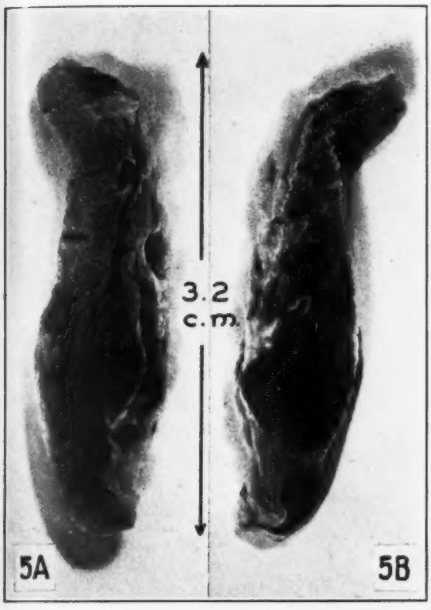
Plate 60

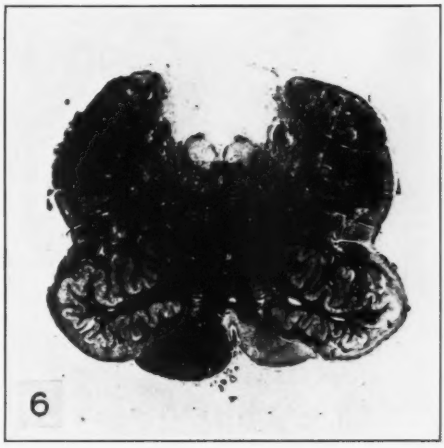
Fig. 5*a*. Spleen showing siderofibrotic changes with marked wrinkling of the capsule.

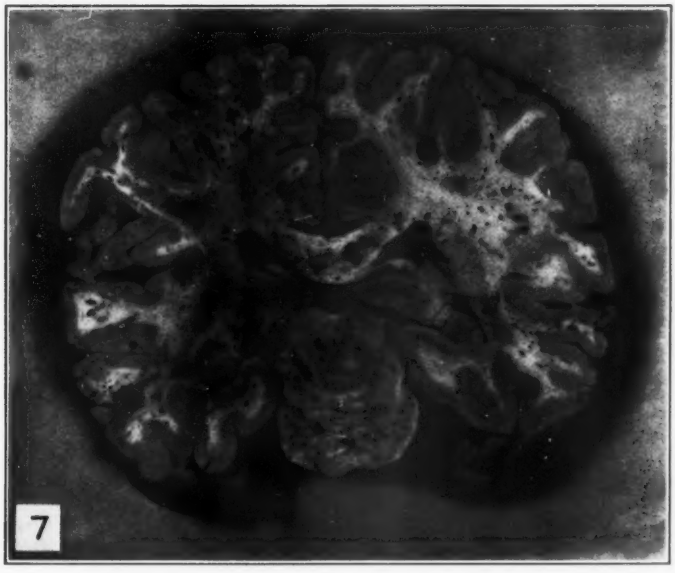
Fig. 5*b*. Section of spleen showing large area of hemorrhage and greatly scarred pulp.

Fig. 6. Microphotograph of a section of the medulla showing marked difference in size of the right and left pyramidal tracts, the former being markedly degenerated.

Fig. 7. Cross section of adult brain showing multiple petechial hemorrhages throughout.





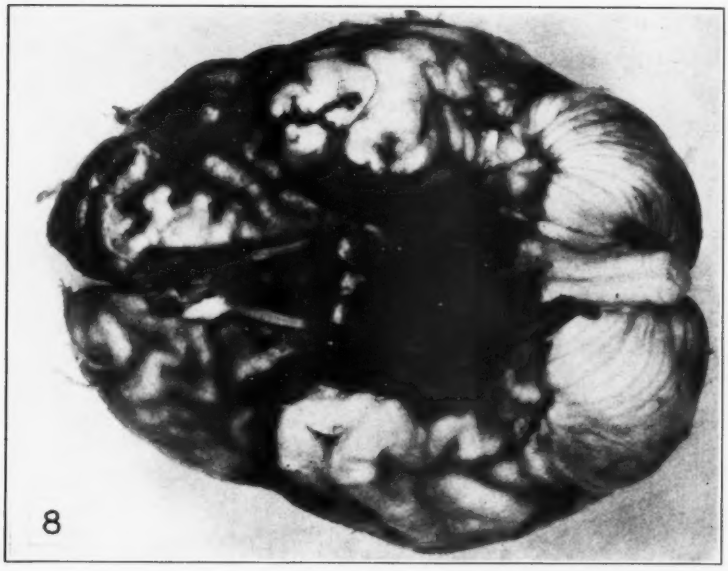


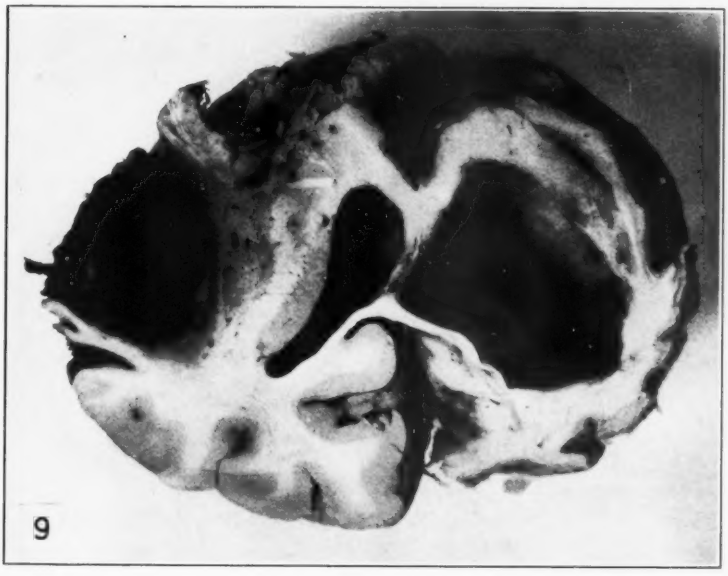
Bridgers Cerebral Vascular Disease in Sickle Cell Anemia

PLATE 61

FIG. 8. Case 1. Inferior surface of brain showing subarachnoid hemorrhage in the form of a blood clot with inequality in the size of the frontal lobes.

FIG. 9. Case 1. Cross section of posterior aspect of brain showing marked atrophy of left cerebral cortex, dilated ventricles, petechial hemorrhages and a large hemorrhage in the right hemisphere.





Bridgers Cerebral Vascular Disease in Sickle Cell Anemia

PATHOLOGICAL CHANGES FOLLOWING THERAPEUTIC HYPERTHERMIA *

Report of a Case

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In view of the current vogue that therapeutic hyperthermia enjoys for a wide variety of ailments, it is appropriate to take stock of the more serious complications that may follow its use even under proper management as a hospital procedure and with careful selection of patients. In a preliminary survey [1] of hyperpyrexia produced by physical agents, the Council on Physical Therapy of the American Medical Association stated in 1934 that 29 deaths following fever treatment had been reported by physical therapists in response to its questionnaire. Since then at least 16 additional fatalities have been reported.[2-12] Moreover, there is good reason to believe that there have been a considerable number of other fatal accidents which for one reason or another have not been recorded in the current literature. Although it is in only a few of these cases that complete autopsies have been performed, sufficient data have accumulated to permit a satisfactory discussion of at least the morbid anatomical changes that may result from fever therapy, by whatever physical means induced. While there appears to be considerable variation in the changes observed in individual cases, certain structures, notably the brain, liver and blood vessels, seem particularly susceptible to injury of a type to be described presently, and with sufficient frequency to be regarded as characteristic.

The writer has had occasion to perform an autopsy on a patient who developed uncontrollable hyperpyrexia (109° F.), coma, and respiratory failure in the course of the third of a series of hyperthermia treatments for infectious arthritis of the fingers. The patient died approximately 35 hours after fever was initiated. The method of inducing hyperthermia was that of hot bath followed by blanket pack. The clinical course will be related in some

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detail, not only because it is dramatic but because it is essential to the interpretation of the pathological changes that ensued. The fact that the patient was a comparatively young man of good general physique and in good health, except for the arthritis, makes it possible to ascribe the pathological changes observed to the hyperthermia *per se*. The changes found at autopsy are of particular interest, furthermore, in that they demonstrate certain interesting vascular lesions occurring in the brain, kidneys and spleen, hitherto undescribed in connection with fatalities following fever therapy.

Report of Case *

Clinical History: The patient, a white male 36 years of age, robust of build and in good general health, was admitted to the Hospital for Joint Diseases on Sept. 25, 1936, in order to receive fever therapy for infectious arthritis of the fingers of undetermined etiology. He had had 2 previous treatments for the same complaint without ill effect and with some symptomatic improvement. The method of inducing hyperthermia was that of hot bath followed by blanket pack. The physical examination done before treatment was essentially negative except for the arthritis. The blood pressure was 112 systolic, 80 diastolic.

Fever treatment using bath and blanket pack was begun at 1 P.M., the temperature being raised from 97.8° F. (initial) to 105.2° F., by mouth, within 35 minutes. The patient's pulse varied from 134 to 148, and the temperature fluctuated between 104.6° F. and 105° F. for the next 4 hours. One ¼ grain dose of morphine was given at 1.45 P.M. and this was repeated at 5.30 P.M. because of excessive excitement. At 6.15 P.M. the temperature suddenly rose to 106° F. (pulse 138). At 6.30 P.M., with the temperature still at 106° F., the patient was uncovered to expose both shoulders, but the temperature nevertheless continued to rise steadily to 107.4° F. within the next 15 minutes. Treatment was stopped immediately; the patient was rapidly unpacked and received coramine hypodermically by way of stimulation. The temperature dropped to 106.2° F. but again rose promptly to 107° F. (pulse 168) and despite cool sponging continued to climb to 109° F. (rectal). An ampule of sodiocaffeine benzoate was administered intravenously. At 7 P.M. the patient lapsed into unconsciousness and his pulse was rapid. By 7.25 P.M., after a cool enema containing 30 grains of aspirin, the temperature dropped sharply to 100° F. (rectal). At 7.35 P.M. the temperature again rose to 104.4° F. (rectal). At 8 P.M. an intravenous hyperdermoclysis of normal saline was begun. The temperature fluctuated between 100° F. and 105° F. during the next 2 hours. Caffeine and coramine were again administered. The patient vomited about 8 ounces of dark brown material. A Levine tube was passed to evacuate the stomach and gastric lavage was instituted with 5 per cent sodium bicarbonate. At 10.35 P.M. the patient's respirations stopped but he responded to artificial respiration. The patient again received caffeine and coramine intravenously, intracardiac injection of adrenalin, and also oxygen and carbon dioxide by nasal catheter.

* The writer wishes to express his indebtedness to Dr. M. Dinnerstein for permission to include the clinical data in this case.

With the aid of these emergency supportive measures, the patient lasted through the night. At 5 A.M. the next morning, with the patient in an oxygen tent and still comatose, clonic twitchings of the left lower extremity were observed and it was noted that he had a right hemiplegia, including the face, of flaccid type. At 10 A.M. that morning he was seen by a neurological consultant, who tapped about 20 cc. of spinal fluid under increased pressure. Laboratory examination of this fluid showed increased globulin content and a cell count of 14 white blood cells and 9 red blood cells per cmm. The spinal fluid Wassermann was negative. The urine specimen at this time contained albumin (++), casts, leukocytes, and an occasional red cell.

Despite repeated stimulation with coramine, adrenalin, digitalis and caffeine, the patient remained stuporous and finally expired at 12.20 A.M. (Sept. 27, 1936), 35 hours after hyperthermia was started and approximately 29 hours after the onset of coma and collapse.

Postmortem Examination

Autopsy was performed 2 hours after death. The body was that of a robust, well developed adult white male. The body was still warm and rigor mortis had not yet set in. External examination did not reveal any obvious swellings of the finger joints. There was marked purplish mottling of the face and trunk, and considerable postmortem lividity. No jaundice or edema was noted.

The panniculus adiposus was moderately thick. The peritoneal surfaces were everywhere smooth and glistening, except for some fibrous bands extending from the cecum to the anterior abdominal wall at the site of an old appendectomy scar. The intestines showed no distention whatsoever and no evidence of hemorrhage. The diaphragm on the left side appeared to be somewhat elevated, apparently in consequence of gastric dilatation.

There was no fluid in the pleural cavities. The upper lobes of the lungs felt hypercrepitant, while the lower lobes, particularly the left, showed large purplish areas of atelectasis. On section the lungs had a mottled, dark reddish purple appearance and exuded an inordinately large amount of venous blood. There was no gross evidence of bronchopneumonia, although the bronchi were somewhat congested and contained frothy pinkish fluid. The pulmonary arteries and veins appeared normal.

The pericardium contained a small amount of clear fluid. The heart showed slight enlargement due to dilatation of its chambers. The valves showed nothing unusual except for occasional lipoid plaques. The myocardium was firm and dark red in color. There

were a few subendocardial petechial hemorrhages in the left ventricle. The ostia and lumens of the coronary arteries were widely patent.

The liver was slightly enlarged, soft and smooth, and was a yellowish brown color. On section the lobular architecture was found clearly outlined. Scattered throughout the liver were small, focal yellowish areas which appeared to be fat, but which were nevertheless somewhat suggestive of focal areas of necrosis. The gall bladder was slightly distended. The bile ducts and portal veins showed nothing noteworthy.

The spleen was soft and somewhat enlarged. There was a circumscribed, slightly elevated subcapsular area of infarction, about 2 cm. in diameter, on its medial aspect. The splenic pulp had a dark red appearance and could not be scraped away easily. The malpighian follicles were quite distinct.

The pancreas showed nothing noteworthy.

The adrenals were of average size. They presented a yellow lipoid cortex and some autolysis of the medulla.

The kidneys were of normal size and their capsules stripped with ease revealing smooth, slightly lobulated reddish surfaces. The latter showed several dark red mottled areas, about 1 cm. in diameter, which on section appeared to be fresh infarcts. The cortex and medulla had indistinct markings. The ureters and urinary bladder presented nothing unusual. The prostate was small and on section showed no evidence of inflammation.

The stomach was moderately distended and contained turbid dark fluid. The gastric mucosa was markedly congested and revealed a few petechial hemorrhages but no gross evidence of gastritis. The duodenum was not unusual in appearance. The remainder of the gastro-intestinal tract presented nothing striking, *i.e.* no evidence of ulceration, necrosis or hemorrhage.

On removal of the calvarium the brain was noted to be voluminous and quite heavy. The inner aspect of the dura had a thickened, reddish hemorrhagic appearance. The sulci of the cerebral cortex were somewhat flattened and the surface of the hemispheres showed intense venous engorgement and considerable edema. There were also several superficial reddish areas of hemorrhage, about 1 to 2 cm. in diameter, noted beneath the pia.

The cortex of the cerebral hemispheres was edematous, quite

soft and friable. Section of the brain revealed innumerable focal punctate hemorrhages distributed throughout the gray matter of the cortex. These were sharply limited to the cortical gray matter and were most prominent in the depths of the sulci (Fig. 1). They frequently involved several contiguous sulci, but on the whole their distribution was distinctly patchy. The punctate hemorrhages were more extensive in the left hemisphere than in the right. Microscopic sections indicated that most of the hemorrhagic areas still contained well preserved red cells, but in places one could observe beginning laking with blood pigment deposits (Fig. 2). There were no hemorrhagic lesions in the white matter of the hemispheres. In the left internal capsule, however, there was an irregular area of hemorrhage and softening approximately 1 to 2 cm. in diameter (Fig. 4). The pons, medulla and cerebellum were sectioned serially, but no evidence of hemorrhage, softening or conspicuous edema was observed. The spinal cord could not be examined.

Anatomical Diagnoses: Status (35 hours) after therapeutic hyperthermia for arthritis: multiple punctate hemorrhages in gray matter of cerebral cortex; hemorrhage in left internal capsule; thrombosis of venules and capillaries in cerebral cortex and internal capsule; cerebral congestion and edema; focal necrosis of interlobular arteries of kidneys and splenic artery branches; infarcts of kidneys and spleen; severe hepatic degeneration and edema; subpleural and subendocardial petechial hemorrhages; pulmonary congestion, hemorrhage and edema; and partial atelectasis of the lower lobes of both lungs.

Microscopic sections confirmed the gross changes as outlined in the anatomical diagnoses. The significant histological observations will be indicated in connection with the ensuing discussion.

Discussion

The following discussion of the anatomical changes occurring after therapeutic hyperthermia is based largely on the present writer's own observations, but also on the information afforded by 9 autopsies reported within the past few years. These published autopsy reports [2-4, 8, 9, 11, 12] have, in most instances, been presented briefly, often without important details or illustrative microphotographs, but they serve nevertheless to indicate in a

general way the principal complications and sequelae of therapeutic hyperpyrexia.

Pathological Changes in the Brain: It is usually in the brain that the most extensive and consequential damage is encountered after therapeutic hyperthermia. To judge by the recorded fatalities, cerebral edema and conspicuous congestion of the meningeal vessels are constantly present. In our case also, the brain was swollen and quite heavy and the surface of the hemispheres showed intense venous engorgement. There was also a pressure conus of the cerebellum.

Another frequent observation is meningeal hemorrhage, which seems to be attributable to extreme venous and capillary vasodilatation. Specifically, rupture of engorged venules probably occurs, with consequent perivascular hemorrhages. Thus, the inner aspect of the dura may have a thickened, diffusely hemorrhagic appearance, or on the other hand, the subdural hemorrhages may be less prominent and focal in distribution. Small extravasations of blood beneath the arachnoid and pia mater have also been noted. Equally conspicuous and of greater significance is the presence in our case of innumerable punctate hemorrhages, irregularly distributed throughout the gray matter of the cerebral cortex, as illustrated in Figure 1. Microscopic examination shows most of these extravasations of blood to be situated around congested venules and capillaries. Perivascular hemorrhages have also been described [2, 11] in the basal ganglia and in the cerebellum. Moreover, hemorrhage and softening may occur in the internal capsule (explaining the development of hemiplegia) as in the case reported here. Histological examination of sections of the hemorrhagic portion of the internal capsule reveals numerous perivascular blood extravasations, many of which are limited to the Virchow-Robin spaces while others appear to be somewhat more extensive (Fig. 4).

Associated with these hemorrhagic phenomena there is pronounced cellular degeneration and even necrobiosis in the gray matter of the cerebral cortex. Microscopically the nerve cells of the more hemorrhagic portions of the cortex (Fig. 2), in contrast with the relatively well preserved zones (Fig. 3), show considerable alteration. Some nerve cells reveal ischemic changes, while others are shrunken and deeply pigmented. Indeed, many cells

have completely disappeared, leaving wide bands of necrobiosis which terminate more or less abruptly where the preserved cortex begins again. In connection with the shrinkage of cerebral cortical cells after hyperthermia there is, as Yannet and Darrow [13] have shown, a loss of intracellular fluid into the extracellular tissue, resulting in increased concentration of intracellular electrolytes.

Furthermore, there were vascular lesions in our case within the hemorrhagic portions of the cerebral cortex and internal capsule, which in themselves are of considerable interest. Microscopic examination of sections of the affected portions of the cortical gray matter reveals a number of thrombosed venules which show evidence of severe injury, if not actual partial necrosis of their walls, as indicated by loss of normal structure and continuity, as well as by intramural and perivascular infiltration with polymorphonuclear leukocytes. The lumen of the venule shown in the accompanying microphotograph (Fig. 5) is partially filled with homogeneous material possessing the staining properties of fibrin. Numerous small venules and capillaries in the hemorrhagic area of the internal capsule reveal similar evidence of injury. In view of the intramural leukocytic reaction, these vascular lesions must be regarded as occurring ante mortem. However, they cannot be held entirely responsible for the cellular changes in the cortical gray matter, since fairly extensive necrobiosis of cortical nerve cells has been noted following therapeutic hyperthermia without the presence of fibrin thrombi in venules.

The presence of conspicuous hemorrhages in one or another part of the brain has led some observers to describe the condition of this organ erroneously as a hemorrhagic encephalitis. The use of the term encephalitis in this connection is inaccurate, since microscopic examination does not disclose any evidence of inflammation associated with the hemorrhages. Our knowledge of the subject would be more enhanced if, in the future, the actual pathological changes in the brain in any given instance were described objectively in some detail, rather than an attempt made, for the sake of convenience, to pigeonhole them in a category to which they do not belong.

In our case a history was elicited of an alcoholic excess prior to hyperthermia treatment, and that naturally raises the question of the possible contributory rôle of alcohol in the production of

cerebral injury following hyperpyrexia. This possible association is further suggested by the observation, well known in large municipal hospitals, that heat stroke is prone to develop in moderate or heavy drinkers. Of course, alcoholism *per se* does not give rise to the cerebral lesions described in the case reported here. However, chronic alcoholism is known [14] to be capable of producing cerebral edema, degeneration of cortical ganglion cells, and endothelial injury sometimes resulting in petechial hemorrhages in the dura and in the midbrain. Acute alcoholic intoxication may likewise cause cerebral edema and also marked vasodilatation. It seems justifiable therefore to add alcoholism to the list of more obvious contra-indications which make artificial fever therapy hazardous: namely, advanced age or marked debility, cardiovascular disease, renal insufficiency, tuberculosis, diabetes, and so on. Furthermore, it seems a reasonable precaution to advise patients receiving a course of hyperthermia treatments to refrain from alcoholic indulgence during the interval preceding each treatment.

Vascular Lesions in Other Organs: In addition to the vascular lesions described in the brain there were also changes in the kidneys and in the spleen. Such lesions have apparently not been observed by others who have dealt with the question of the pathological changes in these cases. In both kidneys in our case there were multiple, subcapsular, wedge-shaped areas of necrosis, and in the spleen there was a similar area of necrosis. These were indistinguishable from infarcts with respect to location, shape and general appearance, and were not to be confused with the focal areas of toxic necrosis that may occur in the kidneys and other viscera, after severe burns for example. The latter areas are usually irregular in outline, smaller in size, not necessarily peripheral, and have a random distribution without reference to vascular supply. The necroses were apparently not due to embolization, nor was any possible source for emboli found in the heart, aorta or arterial system. Furthermore, the foramen ovale was closed. The blood vessels of the kidneys and spleen moreover showed no evidence of previous thrombosis.

The natural inference therefore is that the renal and splenic infarcts were the result of focal necrosis of interlobular arteries and splenic artery branches respectively. Indeed, that is what was

indicated by the histological sections. Figure 6 (inset) shows a necrotic interlobular artery in the vicinity of one of the renal infarcts. The wall of this artery, higher magnification of which is shown in Figure 7, was necrotic and infiltrated by polymorphonuclear leukocytes. Within its lumen there were irregular aggregates of homogeneous, reddish staining material resembling fibrin and agglutinated erythrocytes. The artery shown in the illustration is probably too small to account for the renal infarct, but it illustrates the type of arterial lesion that must have developed in renal arteries of somewhat larger caliber. Sections of the spleen showed a number of similarly necrotic small splenic artery branches in the vicinity of the infarct. Weigert and Van Gieson stains of affected arteries demonstrated disappearance and disruption of the elastica and collagen fibers. Examination of the pulmonary arteries showed focal areas of altered staining with occasional polymorphonuclear leukocytes within their walls, which suggested vascular injury similar to that observed in the brain, kidneys and spleen, but of lesser extent.

Damage to Liver: Of all the pathological changes observed in fatalities following therapeutic hyperthermia, damage to the liver is secondary in importance only to the injury to the brain. Histological evidence of degeneration of varying degree in the liver appears to be frequent. In connection with the more severe reactions necrosis may develop, and indeed this was noted in 4 [3, 8, 12] of the 6 reported fatalities in which the status of the liver was mentioned. Also in the case herein described the liver cells showed microscopically extensive vacuolization and hyaline droplet degeneration (Fig. 8). Areas of necrobiosis were likewise present, as evidenced by pyknosis and disappearance of nuclei, with effacement of cytoplasmic detail. A sudan III stain for fat showed the liver cells to contain numerous fat droplets. In addition to these degenerative changes there was also hepatic edema, as indicated by separation of the sinusoidal walls from the liver cell cords in many places and the presence of granular material within these pericapillary spaces.

That damage to the liver may also occur in non-fatal reactions to therapeutic hyperthermia is indicated by the reports of jaundice [9, 15, 16] developing as a complication of treatment. Warren and and coworkers,[16] for example, noted the appearance of jaundice

after prolonged fever therapy in 7 patients. One may reasonably inquire whether some of these individuals may not subsequently develop nodular cirrhosis of the liver, but that question cannot as yet be answered.

Pulmonary Changes. The usual anatomical changes in the lungs after fatal reactions to therapeutic hyperthermia are marked congestion, edema and alveolar hemorrhage. These changes were noted in most of the available reports in which the lungs were described histologically. In our case, too, microscopic examination showed extreme engorgement of the pulmonary arteries and alveolar capillaries, as well as extravasation of blood into the alveoli, apparently as a result of capillary rupture. These findings are apt to be more pronounced in the lower lobes, where hypostasis also favors development of partial atelectasis. In patients who survive for several days or more bronchopneumonia tends to develop in the hemorrhagic areas.

Changes in the Suprarenal Glands: The published reports of hyperthermia fatalities in which the status of the adrenals is given do not indicate that there is any significant injury to the suprarenal glands. Also in the case reported here there were no striking histological changes. It should be mentioned, however, that hemorrhage into the suprarenals has been noted [2] and that degenerative changes in the adrenal cortex have been described.[2, 9] The latter, however, have not been clearly distinguished from postmortem changes, which may proceed rapidly in the suprarenal glands after febrile states.

Pathological Changes in Other Organs: To complete this résumé of the pathological changes following therapeutic hyperthermia, the changes noted in the other organs will be briefly mentioned. The heart often shows epicardial or endocardial petechial hemorrhages. The kidneys may show hyaline droplet degeneration of the epithelium of the convoluted tubules, hyaline tubular casts, and conspicuous congestion of the glomerular tufts. The spleen in the case reported here showed, microscopically, intense hyperemia of the pulp and fibrin thrombi within pulp venules. In the gastro-intestinal tract dilatation of the stomach as well as congestion and petechial hemorrhages of the gastric mucosa were noted. Hemorrhages in the jejunum and ileum have also been described.[9] Severe gastro-intestinal hemorrhage has been observed in a patient

who died of acute necrosis of the liver following therapeutic hyperthermia.[12]

It should be pointed out that the extent and distribution of the pathological changes in any given instance depend not only on the severity of the injury, but also on the length of the survival period. For example, the histological changes described in the brain take more than a few hours to develop. Moreover, if the cerebral insult is so great that it leads rapidly to fatal edema of the brain, one may not have the opportunity of observing changes in the liver or the vascular lesions which have been described. Thus, in an interesting case cited by Kopp and Solomon,[9] jaundice did not manifest itself until the 3rd day following hyperthermia. By the same token the pathological changes described in the hitherto reported fatalities may not necessarily represent a complete picture of the possible complications and sequelae of hyperthermia. As the survival period is lengthened and as more pathological material is studied, other evidences of tissue injury and response may manifest themselves.

Comment

In attempts to explain the pathogenetic mechanism of the changes that have been noted after therapeutic hyperthermia, various hypotheses have been suggested. Hartman [11] maintains from his own experimental data and also on theoretical grounds, that anoxemia is a paramount factor in the development of cerebral injury after hyperpyrexia treatment. However, it is questionable whether it is correct to attribute the cerebral damage entirely or even largely to anoxemia, to the exclusion of other, probably more weighty factors. Another hypothesis, particularly stressed by Kopp and Solomon,[9] is that of vasomotor shock or collapse. Still other observers have pointed out that the pathological changes bear some similarity to those observed in individuals who succumb to heat stroke. Dehydration, anhydremia and electrolyte depletion are all physiological effects of severe hyperthermia, which may influence and modify the clinical course. The use of certain sedative drugs, for example sodium amytal and morphine, has also been suggested as a possible contributory factor. Furthermore, the convulsions that frequently occur in severe hyperthermia reactions may be responsible for cerebral hemor-

rhages. It may readily be seen therefore that the problem of explaining the pathological changes that may follow therapeutic hyperthermia induced by physical means is a complex one owing to the interplay of many factors whose relative importance is difficult to evaluate.

A note of caution is decidedly in order with regard to the serious but not necessarily fatal complications of therapeutic hyperthermia. Physical therapists conducting active fever therapy services generally admit [9, 17, 18] to an appreciable number of patients who in the course of treatment go into so-called "shock" or "circulatory collapse," associated usually with uncontrolled hyperpyrexia, but whom they are able to resuscitate by immediate cessation of treatment and emergency supportive measures. These patients frequently manifest delirium, mania, disorientation, convulsions, epileptiform seizures or coma. They may even develop loss of bladder and rectal sphincter control, transient hemiplegia, facial palsy or aphasia. The exponents of fever therapy are inclined to minimize the significance of such untoward episodes. Even neurologists and psychiatrists [19] are prone to pass lightly over these manifestations of brain injury as transitory neurological changes. The possibility, however, that such individuals may actually sustain organic lesions in the brain, and perhaps in other viscera, cannot be dismissed. Possibly, therefore, our attitude should be a more guarded one for the present, pending follow-up observation of such individuals for hyperthermia sequelae.

Summary

A description is given of the changes observed at autopsy in a case of uncontrollable hyperpyrexia (109° F.) ensuing upon hyperthermia treatment for arthritis of the finger joints. The hyperpyrexia (which developed in the course of the third of a series of treatments) was associated with coma and respiratory failure, and the patient died about 35 hours after the fever was initiated. In this case the significant pathological changes were the following: (1) multiple punctate hemorrhages and necrobiosis in the gray matter of the cerebral cortex; (2) hemorrhage in the left internal capsule; (3) thrombosis of venules and capillaries in the cerebral cortex and internal capsule; (4) cerebral congestion and edema; (5) infarction of kidneys and spleen; (6) marked

hepatic degeneration and edema; and (7) pulmonary congestion, hemorrhage and edema.

The changes found in this case have been correlated with, and discussed in relation to, those in 9 cases previously recorded in the literature. Certain of the changes seen in the case reported here, notably the vascular lesions, have not hitherto been described in connection with fatalities following fever therapy. Specifically there seems to be no previous description of the thrombosis of venules and capillaries in affected portions of the brain, and of the infarcts in kidneys and spleen, apparently due to focal necroses of small arterial branches of these organs.

The principal complications and sequelae of hyperthermia — and especially its effects upon the brain, blood vessels and liver — are indicated. Attention is also drawn to the fact that the reactions to therapeutic hyperthermia are sometimes serious even when they are not fatal.

REFERENCES

1. Council on Physical Therapy. Hyperpyrexia produced by physical agents. *J. A. M. A.*, 1934, **103**, 1308–1309.
2. Hartman, F. W., and Major, R. C. Pathological changes resulting from accurately controlled artificial fever. *Am. J. Clin. Path.*, 1935, **5**, 392–410.
3. Watts, F. Abstracts Fifth Annual Fever Conference, Dayton, Ohio, 1935, 75.
4. Schnabel, Truman G., and Fetter, Ferdinand. Fever therapy in gonorrheal arthritis and chorea. *Ann. Int. Med.*, 1935, **9**, 398–405.
5. Simpson, W. M. Discussion in Abstracts. Fifth Annual Fever Conference, Dayton, Ohio, 1935.
6. Hench, P. S. Clinical notes on the results of fever therapy in different diseases. *Proc. Staff Meet. Mayo Clin.*, 1935, **10**, 662–666.
7. Desjardins, Arthur U. Fever therapy. *Arch. Phys. Therapy*, 1936, **17**, 206–214.
8. Wilbur, E. L., and Stevens, J. B. Morbid anatomic changes following artificial fever, with report of autopsies. *South. M. J.*, 1937, **30**, 286–290.
9. Kopp, I., and Solomon, H. C. Shock syndrome in therapeutic hyperpyrexia. *Arch. Int. Med.*, 1937, **60**, 597–622.
10. Neymann, C. A. Discussion. *Arch. Phys. Therapy*, 1936, **17**, 215.
11. Hartman, F. W. Lesions of the brain following fever therapy — etiology and pathogenesis. *J. A. M. A.*, 1937, **109**, 2116–2120.

12. Chunn, G. D., and Kirkpatrick, C. L. Fatal result of artificial fever therapy — a case report. *Mil. Surgeon*, 1937, 81, 281–287.

13. Yannet, H., and Darrow, D. C. The effect of hyperthermia on the distribution of water and electrolytes in brain, muscle and liver. *J. Clin. Investigation*, 1938, 17, 87–94.

14. Weil, Arthur. Textbook of Neuropathology. Lea and Febiger, Philadelphia, 1933, 190.

15. Horowitz, E. A. Hyperpyrexia or sulfanilamide in the treatment of gonorrhea in women. *M. Clin. North America*, 1938, **22**, 1429–1441.

16. Warren, Stafford L., Scott, Winfield W., and Carpenter, Charles M. Artificially induced fever for the treatment of gonococcic infections in the male. *J. A. M. A.*, 1937, **109**, 1430–1434.

17. Stecher, Robert M., and Solomon, Walter M. The treatment of gonorrheal arthritis with artificial fever. *Am. J. M. Sc.*, 1936, **192**, 497–510.

18. Stecher, Robert M., and Solomon, Walter M. The complications and hazards of fever therapy: analysis of 1000 consecutive fever treatments with the Kettering hypertherm. *Ann. Int. Med.*, 1937, **10**, 1014–1020.

19. Ebaugh, F. G., Barnacle, C. H., and Ewalt, J. R. Psychiatric aspects of artificial fever therapy. *Arch. Neurol. & Psychiat.*, 1938, **39**, 1203–1212.

DESCRIPTION OF PLATES

PLATE 62

FIG. 1. Photograph of section through the left cerebral hemisphere showing multiple punctate hemorrhages limited to portions of the gray matter of the cortex. Note also the hemorrhagic appearance of the leptomeninges.

FIG. 2. Microphotograph of hemorrhagic area in gray matter of cerebral cortex showing numerous small perivascular extravasations and extensive cellular degeneration. Toluidine blue stain. × 125.

FIG. 3. Microphotograph of relatively well preserved area of cortical gray matter for contrast with Fig. 2. Note engorgement and vasodilatation of meningeal vessels. Toluidine blue stain. × 125.

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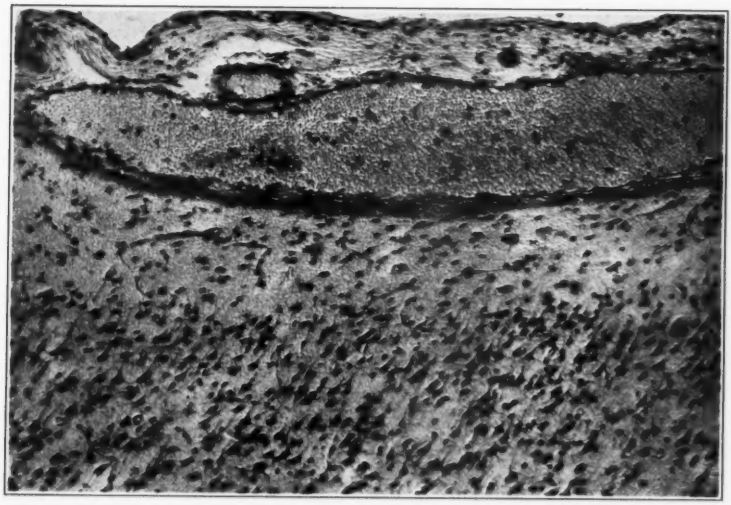

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PLATE 63

FIG. 4. Microphotograph of an area of hemorrhage and softening in the left internal capsule. Note the perivascular extravasations and the thrombosed venule. × 85.

FIG. 5. Microphotograph of venule in hemorrhagic portion of cortical gray matter, showing fibrin thrombus and intramural infiltration with polymorphonuclear leukocytes. × 250.

FIG. 6. Microphotograph of one of the renal infarcts. Note the necrotic interlobular artery in inset. × 25.

4

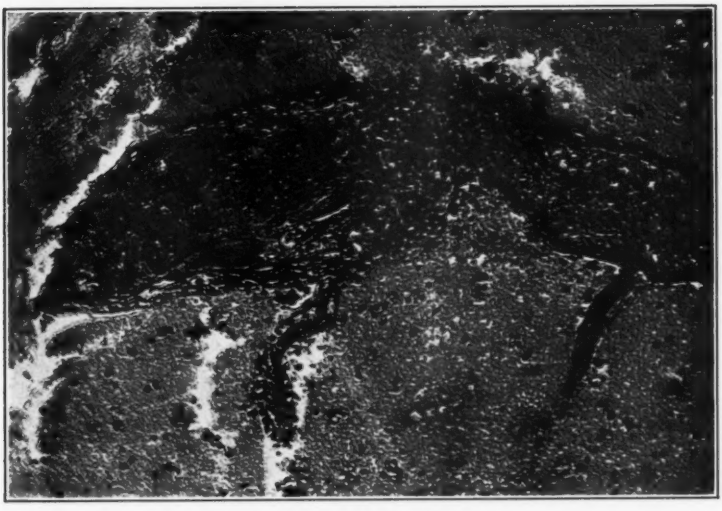

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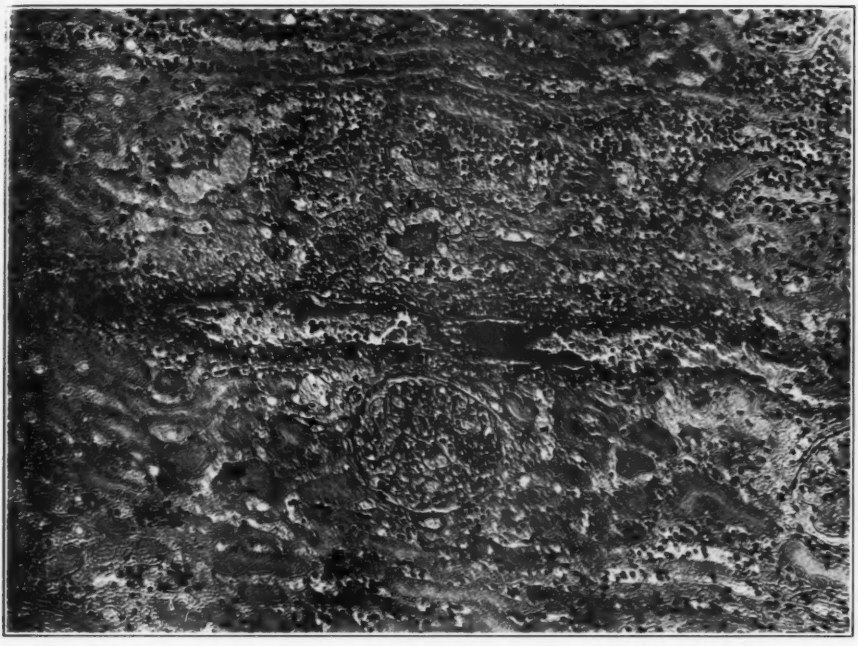
PLATE 64

FIG. 7. Higher magnification of necrotic interlobular artery illustrated in Fig. 6. × 100.

FIG. 8. Microphotograph of liver showing severe degenerative changes of liver cells and evidence of hepatic edema. × 600.



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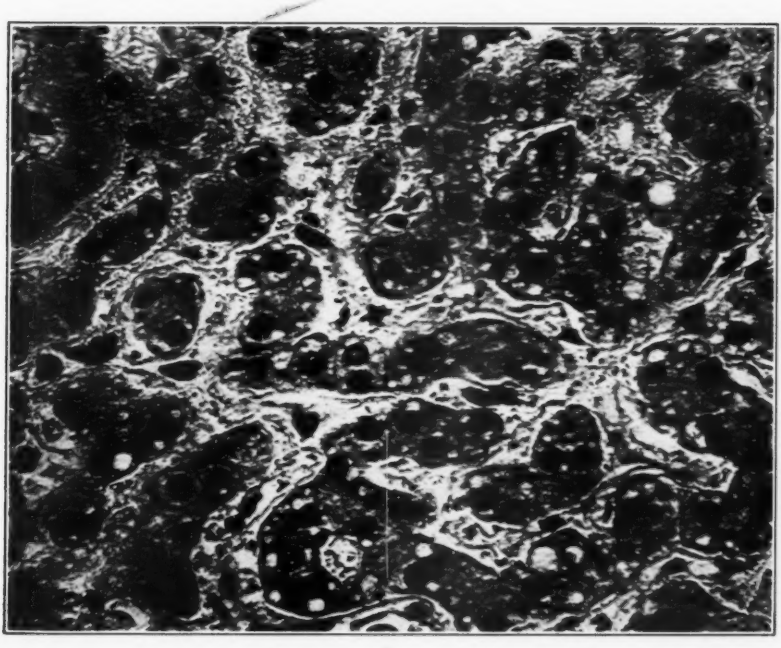

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THE LYMPHOID NODULES OF HUMAN BONE MARROW *

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REVIEW OF THE LITERATURE

A survey of the literature shows that Dominici [1] in 1902 observed focal collections of lymphocytes in the bone marrow. Longcope [2] described these nodules in the marrow as a characteristic occurrence in typhoid fever and also noted small foci in 2 cases of pneumonia. Hedinger [3] demonstrated a number of large follicles with large germinal centers in the femoral marrow of a 1½ year old idiot with so-called "status lymphaticus." Dickson [4] stated that nodules of small lymphocytes are scanty in normal marrow. Oehme,[5] in a series of 23 cases of rickets, and in 2 other cases, described, in otherwise normal appearing red marrow, follicles with germinal centers in 12 cases and others without germinal centers in 2 cases. The majority of Oehme's cases were infectious and all were infants or children. He was undecided as to whether these follicular structures were normal or not. Such a high incidence of follicles with germinal centers has not as yet been confirmed.

In 1915 Askanazy,[6] in a study of the marrow from different regions of the femur in a series of 126 unselected cases, most of which were adults, described scattered lymphoid nodules in 33 per cent. These nodules varied in size from 201 to 542 microns in diameter. They were composed mostly of small lymphocytes, intermingled at times with myeloid cells, and each follicle had a reticular framework with a central or eccentric arterial capillary. Only 1 case presented germinal centers and local lesions did not account for the presence of the foci of lymphocytes. These foci were more frequent in adult individuals over 40 years of age. The author concluded that lymphoid nodules in the bone marrow cannot be considered abnormal but that an excessive number and many germinal centers may be a pathological phenomenon.

Von Fischer,[7] in a detailed histological study of the marrow of

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the humerus in a series of 61 unselected cases, and from the ribs and vertebra in 11 of the same series of cases, confirmed and extended Askanazy's findings. Her cases were mostly in adults. She emphasized the association of these lymphoid nodules with active red marrow. It is to be noted that she found lymphoid nodules in the vertebral marrow in 9 of the 11 cases in which this marrow was studied. Mayer and Furuta [8] demonstrated lymphoid nodules in 15 per cent of 58 essentially morphologically different cases and agreed with Askanazy and von Fischer as to their significance. It is to be noted that infection and inflammatory lesions in the various organs are frequent in such varied material as that studied by the latter authors, as will be pointed out later.

Ziegler,[9] Karsner [10] and others described focal aggregations of lymphocytes in the marrow in pernicious anemia. Custer,[11] Darling and co-authors [12] and others reported them to be present in cases of agranulocytosis. Custer, in commenting on the dense focal accumulations of lymphocytes in the marrow in 9 of his 11 cases of agranulocytosis, on the basis of the presence of considerable numbers of large, pale, delicately staining cells in the central portion of the lymphoid masses, wondered whether they were true lymph follicles or merely folliculoid accumulations in response to focal degeneration. He stated that the nodules are seen infrequently in the marrow in other types of disease.

Materials and Methods

The material for this study consisted of a series of 265 unselected cases. All were adult individuals. Those showing primary diseases of the hemopoietic system were excluded. Marrow from the vertebra, sternum, humerus, femur and tibia in the first 122 cases, and from the vertebra and upper third of the shaft of the humerus in the remaining 143 cases, was studied. In addition, marrow from a second series of 23 adults, designated henceforth as the control series, was studied. Only cases with no significant inflammatory lesion demonstrable anywhere in the various organs of the body were selected for this second series. Marrow from the lumbar vertebra and upper third of the shaft of the humerus in all cases was studied. In 11 cases marrow from the lower half of the shaft of the femur was utilized for study. In a strict sense inflammatory lesions could not be considered eliminated from all cases of this

series since, for example, the presence or absence of dental or accessory nasal sinus infections was not commonly confirmed. However, the series as a whole represented one in which the incidence and extent of inflammatory lesions were reduced to a minimum. In 10 of these 23 cases, which were traumatic and where all the individuals died within 6 hours after injury, only the bone marrow was studied histologically. The remaining organs were studied only in gross. All other cases in both series were completely reviewed, both in gross and histologically.

The section technic patterned after the recommendation of Custer [13] was employed. Both hematoxylin-eosin and Maximow's azure II eosin and alum hematoxylin stains were used. In addition to these, consecutive sections from the same blocks of tissues from selected cases were stained with Foot's modification of Bielschowsky's method for reticulum and Mallory's phosphotungstic acid hematoxylin method for fibrin.

Histological Examination

Since the lymphoid nodules studied in each series were quite similar, they may be described together. They were roughly oval in shape and at low magnification stood out in sharp contrast with the surrounding areas of granulopoiesis and erythropoiesis (Fig. 1). In addition to the oval shaped nodules, other areas appeared in sections as elongated structures with narrow diameters. The margins were slightly irregular and extended outward for short distances between adjacent fat cells, merging imperceptibly with the surrounding myeloid tissue. Fat cells were not noted within the nodules. The lymphoid nodules in the first series varied from 560 by 450 microns to 90 by 90 microns. Those in the control series varied from 525 by 450 microns to 90 by 90 microns, and there were also variations between these figures. From 1 to 3 nodules were seen in one low power field, but usually only 1. In a single section only 1 to 4 of these nodules were usually present. Ordinarily they were isolated and widely scattered. Here and there the structures contained capillaries filled with erythrocytes. Many of the nodules in the sections contained a centrally or eccentrically placed blood vessel with an outside diameter of 14 to 20 microns with the thickness of the wall equal to or slightly less than the diameter of the lumen. These were apparently precapillary arterioles.

The cells of the lymphoid nodules were made up almost exclusively of small round cells (Fig. 2), evenly and symmetrically placed, which were morphologically identical with the small lymphocytes of lymph nodes, as determined with the technic employed. A few medium sized to large lymphocytes were usually present. Occasional myelocytes, normoblasts, plasma cells and macrophages were seen, usually at the peripheries of the nodules. Rarely myelocytes were numerous but the lymphoid nodules retained their histological identity. Cells with an oval to fusiform, pale vesicular nuclei and ill defined cytoplasm were scattered here and there. A supporting structure composed of a network of branching argentophil fibrils enclosing groups of cells and occasional single isolated cells was seen (Fig. 3). The nodules were variously located in relation to other structures of the marrow. Occasionally they occurred adjacent to a small artery, venous sinusoid, trabecula of bone, or in the midst of myeloid tissue. The nodules in the material studied were generally located in active marrow, although the hemopoietic cellularity of the marrow at times was slight. They were not found in otherwise wholly fatty marrow.

No activity, as judged by mitosis or increasing number of medium sized and large lymphocytes with increasing basophilia of the cytoplasm, or follicles with germinal centers were seen. There was no constant predominance of any particular cell type in the region at the periphery of the nodules. Other than hyaline and fibrinoid changes within an occasional nodule, there was no evidence that the cells composing the nodules were a response to focal degeneration.

Incidence of Lymphoid Nodules in the Marrow and their Relation to Inflammatory Lesions Elsewhere in the Body

Table I shows the incidence of inflammatory lesions in the first

Table I

Relation of Lymphoid Nodules in the Marrow to Inflammatory Lesions

Material	Total number of cases	Number of cases with inflammatory lesions	Incidence
			%
Marrow with lymphoid nodules	78	75	96
Marrow without lymphoid nodules	187	169	90

TABLE II

Data from 23 Cases of the Control Series

Number of case	Age	Sex	Diagnosis	Degree of hemopoietic cellularity			Lymphoid nodules present	Duration
				V	H	F		
	yrs.			%	%	%		
1	55	F	Traumatic injuries	75	55	..	V. H.	1 hr.
2	Adult	M	Traumatic injuries	70	0–50	0–30	H. F.	1 hr.
3	Adult	M	Traumatic injuries	75	50	0	H.	1 hr.
4	55	M	Traumatic injuries	75	0–50	0–1	V. H.	1 hr.
5	33	M	Traumatic injuries	75	0–25	0–1	F. H.	6 hrs.
6	68	M	Cerebral hemorrhage	5–75	0	..	V.	17 hrs.
7	80	M	Hypertension, chronic cardiac failure	80	0–1	0–1	V.	3 mos.
8	73	M	Cerebral hemorrhage	75	0–25	..	V.	4 hrs.
9	70	F	Traumatic injuries	20–70	0–70	0–15	V. H.	1 hr.
10	52	M	Traumatic injuries	75	0–20	..	—	3 hrs.
11	Adult	F	Traumatic injuries	75	20	..	—	3 hrs.
12	Adult	M	Traumatic injuries	75	0–10	..	—	1 hr.
13	47	F	Traumatic injuries	65	50	..	—	24 hrs.
14	63	M	Traumatic injuries	65	0	..	—	10 hrs.
15	56	M	Traumatic injuries	80	55	0–2	—	24 hrs.
16	47	M	Pontine hemorrhage	75	..	..	—	8 hrs.
17	50	M	Cerebral hemorrhage	75	0–35	0–45	—	3 hrs.
18	Adult	M	Hypertension, chronic cardiac failure	75	0	0	—	3 yrs.
19	Adult	M	Cerebral hemorrhage	75	0–5	..	—	1 hr.
20	35	F	Traumatic injuries	75	10–50	..	—	10 hrs.
21	73	M	Traumatic injuries	10–70	0	..	—	12 hrs.
22	Adult	F	Traumatic injuries	0–60	0–1	0	—	1 hr.
23	65	M	Traumatic injuries	75	0–25	0–1	—	3 hrs.

V = Marrow of vertebra
F = Marrow of femur
H = Marrow of humerus

series of cases with and without lymphoid nodules in the marrow. It is obvious from the data in this table that there is no significant difference in the incidence of inflammatory lesions in the cases with and without lymphoid nodules in the marrow. This does not necessarily rule out association with inflammatory lesions since the nodules may be associated with certain lesions and not with others. Of the 265 cases in the first series, 78 or 29 per cent showed lymphoid nodules in the marrow.

Table II, which includes a brief diagnosis in each case, shows the data on the cases in the control series. Nine, or 39 per cent, showed lymphoid nodules in the marrow. Although the series is small, it may be stated that the nodules occurred frequently in the marrow where the incidence and extent of inflammatory lesions were reduced to a minimum. This is strong evidence against any association with inflammatory disease.

Relation of Lymphoid Nodules in the Bone Marrow to Age

Data was available on the age in 230 cases in the first series of cases. Twenty-eight individuals were 40 years of age, or under, while 202 were older. Lymphoid nodules were present in the marrow in 3, or 10 per cent of the 28 individuals under 40 years of age, and in 64, or 32 per cent of the 202 individuals in the older group. The series of cases in the lower age group is small but when the figures are combined with those of Askanazy [6] and von Fischer [7] the results become significant. It is seen from Table III that in all series the nodules are more frequently found in individuals over 40 years of age than in those younger.

Summary

In summary, it may be stated that lymphoid nodules occur frequently in the bone marrow and that they are definitely associated with active red marrow. They occur in a wide variety of diseases, are frequently found in cases where no infection or significant inflammatory lesion is demonstrable, and are also present frequently in the marrow in cases of sudden death from traumatic injuries. Local lesions in the bone marrrow do not account for these foci. The lymphoid nodules are found more frequently in the marrow in individuals over 40 years of age than in that of adult but younger individuals.

TABLE III

Relation of Lymphoid Nodules in the Bone Marrow to Age

	Askanazy's series	von Fischer's series	Author's series	Total
Total number of cases examined under 40 yrs. of age	44	22	28	94
Number of cases under 40 yrs. of age with lymphoid nodules in marrow	10	10	3	23
Incidence of cases under 40 yrs. of age with lymphoid nodules in marrow	23%	45%	10%	24%
Total number of cases examined over 40 yrs. of age	82	39	202	323
Number of cases over 40 yrs. of age with lymphoid nodules in the marrow	33	28	64	125
Incidence of cases over 40 yrs. of age with lymphoid nodules in marrow	40%	72%	32%	39%

No pathological significance can be attached to the presence in the marrow of lymphoid nodules of the type, size and number described in this report. The evidence appears to justify the concept that the lymphoid nodules are essentially normal though perhaps variable constituents of the active red marrow of adult individuals.

REFERENCES

1. Dominici, Henri. Manuel d'histologie pathologique. V. Cornil and L. Ranvier, Paris, 1902, **2**, 585–691.
2. Longcope, Warfield T. Eine Studie über das Knochenmark bei Typhus und anderen akuten Infektionskrankheiten. *Zentralbl. f. Bakt.*, 1906, **37**, 23–33.
3. Hedinger, Ernst. Über die Kombination von Morbus Addisonii mit Status lymphaticus. *Frankfurt. Ztschr. f. Path.*, 1907–1908, **1**, 527–543.
4. Dickson, W. E. Carnegie. The Bone Marrow. A Cytological Study. Longmans, Green & Co., London, 1908, 37–38.
5. Oehme, C. Lymphfollikel im kindlichen Knochenmark. *München. med. Wchnschr.*, 1909, **56**, 446–449.
6. Askanazy, M. Über die Lymphfollikel im menschlichen Knochenmark. *Virchows Arch. f. path. Anat.*, 1915, **220**, 257–275.

7. Von Fischer, Olga. Über die Lymphknötchen im menschlichen Humerus-, Wirbel- und Rippenmarke. *Frankfurt. Ztschr. f. Path.*, 1917, **20**, 346–380.

8. Mayer, Edmund, and Furuta, S. Zur Frage der Lymphknötchen im menschlichen Knochenmark. *Virchows Arch. f. path. Anat.*, 1924, **253**, 574–586.

9. Ziegler, Kurt. Über die Morphologie der Blutbereitund bei perniziöser Anämie. *Deutsches Arch. f. klin. Med.*, 1910, **99**, 431–467.

10. Karsner, Howard T. Human Pathology: A Textbook. J. B. Lippincott Company, Philadelphia, 1935, Ed. 4, 503.

11. Custer, R. P. Studies on the structure and function of bone marrow. IV. Bone marrow in agranulocytosis. *Am. J. M. Sc.*, 1935, **189**, 507–515.

12. Darling, Robert C., Parker, Frederic, Jr., and Jackson, Henry, Jr. The pathological changes in the bone marrow in agranulocytosis. *Am. J. Path.*, 1936, **12**, 1–11.

13. Custer, R. P. Studies on the structure and function of bone marrow. I. Variability of the hemopoietic pattern and consideration of method or examination. *J. Lab. & Clin. Med.*, 1932, **17**, 951–960.

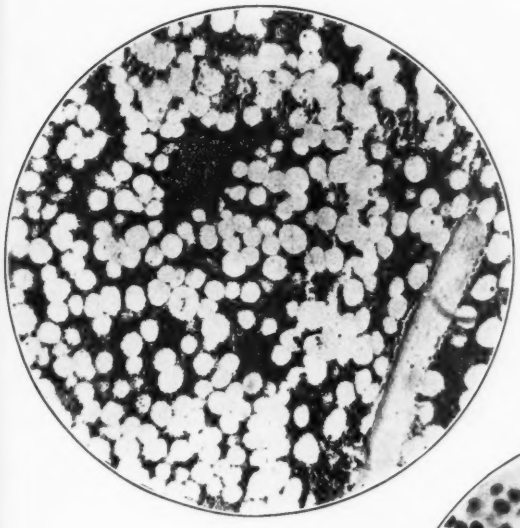
DESCRIPTION OF PLATE

PLATE 65

FIG. 1. In the left upper quadrant there is seen a lymphoid nodule appearing as a unit structure in distinct contrast with the surrounding areas of granulopoiesis and erythropoiesis. Hematoxylin-eosin stain.

FIG. 2. Lymphoid nodule with a centrally placed hyalinized arteriole showing the type cell to be the small lymphocyte. Hematoxylin-eosin stain.

FIG. 3. In the center of the field is seen a lymphoid nodule with a framework of reticular fibers. Foot's modification of the Bielschowsky stain.



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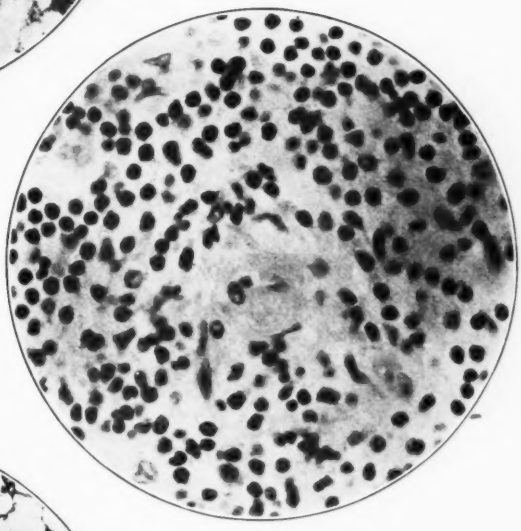

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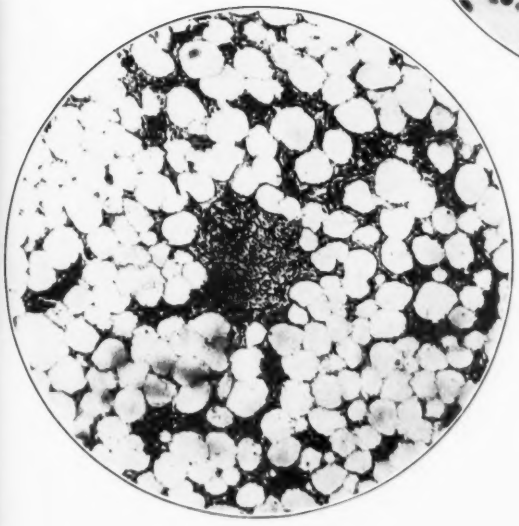

3

STUDIES ON A RAPIDLY DEVELOPING INTESTINAL ADENOMA IN A PIG *

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In a previous publication 3 cases of intestinal adenoma in swine were described.[1] These appeared in herds among which severe intestinal disorders were common. The literature was reviewed and the various etiological concepts were discussed. Regenerative responses of the intestinal epithelium following severe destruction of the intestinal mucosa were also described. Unfortunately these field cases were not brought to our attention until the health of the animals was markedly impaired.

During the course of a nutrition experiment a ration consisting of 50 per cent skim milk powder, 48 per cent yellow corn, and 2 per cent minerals was fed to 1 of 4 groups of swine placed on dry lots. With one exception the pigs fed the above ration grew well throughout the experiment. Some of them were continued on this ration for 10 months. Pig No. 6117 gained in weight until about the 73rd day of the experiment when it reached the peak of its growth. At this time the animal was 105 days old (Chart 1). When weighed 11 days later it had lost 4 pounds, while its litter mate (No. 6118) had gained 20 pounds during the same period. When pig No. 6117 was weighed 6 days later it had lost an additional 4 pounds. There was a gain of only 2 pounds recorded in the next 11 days. One week later it was 8 pounds lighter (Chart 1). Four additional pigs, which were about 1 week older than pigs No. 6117 and No. 6118 when placed on this experiment, were included in this lot. The growth curve of these older pigs so closely resembled that of pig No. 6118 that they were not included in the chart.

About 2 weeks after pig No. 6117 ceased to gain in weight a mild progressive diarrhea was observed. Soon after the onset of the diarrhea small particles of yellowish gray croupous membrane were noted in the feces. The clinical appearance of the pig and the fecal findings were suggestive of a commonly observed form

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of swine enteritis caused by *Salmonella suipestifer* as the primary factor, followed by the secondary invasion by *Actinomyces necrophorus*. The affected pig was removed from the experimental lot and the remaining animals were observed closely for the appearance of similar symptoms. The remaining animals of this lot and

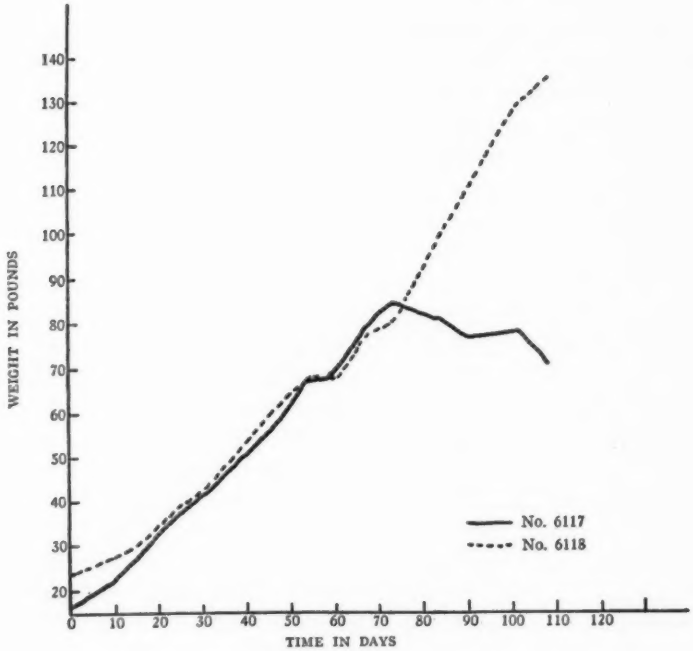


CHART I

of the adjoining lots remained well throughout the duration of the experiment.

Pig No. 6117 became quite weak and on the 108th day of the experiment, at which time the animal was 140 days old, it was destroyed for autopsy. In addition to the non-specific changes in the parenchymatous organs, the mesentery of the small and large intestines was greatly swollen and edematous. The terminal portion of the jejunum and the entire ileum showed numerous elevations on the mucosa. Some of the lesions were low, flattened convex newgrowths, while others were spherical. The ileocecal

valve, cecum and anterior half of the colon were involved in a similar manner. Most of the mucosal elevations were covered with a yellowish croupous membrane that was easily removed by a strong stream of water or by gentle rubbing. When these lesions and the intestinal wall were incised it was found that the newgrowths consisted of a proliferation of the mucosa which did not involve the submucosa. Only a few of the neoplastic formations showed traces of pedicle formation.

Histological study revealed an adenomatous proliferation of the intestinal epithelium. The cells were chiefly of the undifferentiated type, a few goblet cells being found at the outermost edges of the newgrowths. No indication of malignancy was found. Lateral branchings of the newly formed glands were not so well developed as in the previous cases cited.[1] In one section of ileum three acinal formations were found beneath the muscularis mucosa but the basement membrane was intact. The cecal sections of the adenomatous lesions revealed large numbers of *Balantidium coli* which were found in the croupous membrane and in the outermost parts of the proliferating epithelial tissue (Fig. 1). The protozoan forms did not show marked invasive power. The presence of so many *Balantidii* might raise the question of etiological relationship but it should be kept in mind that in swine this protozoan form does not show the same severe invasive power as that of the human. *B. coli* are not found in the small intestine of swine. That the same type of adenomatous formation in the jejunum and ileum failed to reveal *B. coli* offers additional evidence of a lack of etiological relationship of this protozoan form to adenomatosis (Fig. 2).

The growth curve of pig No. 6117 indicates that 30 days prior to the destruction of the pig the adenomatous process had developed sufficiently to impair the health of the animal. In all probability the process began before the pig was 100 days old.

The results of some analyses on the blood of pigs No. 6117 and No. 6118 are shown in Table I. The hematocrit value for pig No. 6118 is well within the normal range, while that of pig No. 6117 is definitely low. This appears to be the result of poor absorption and some loss by exudation, as evidenced by the extensive croupous membrane formation over the adenomatous tissue. In some microscopic fields varying numbers of red cells were present. There was no evidence of gross hemorrhage. The plasma

calcium was low in both pigs while the plasma magnesium for pig No. 6118 was the lowest in the group fed this ration. The high magnesium for pig No. 6117 does not appear to be related to the adenomatosis but more likely it is the result of some dietary factor. The phosphorous partition in the blood of pig No. 6118 was normal, while that of pig No. 6117 showed a high inorganic and a low lipid phosphorus. Pig No. 6117 was undoubtedly metabolizing its own body proteins. Normal pigs when fasted do

TABLE I

Blood Analyses

	Pig No. 6117		Pig No. 6118	
	Whole blood	Plasma	Whole blood	Plasma
Hematocrit value	27		48	
	mg./100 cc.	*mg./100 cc.*	*mg./100 cc.*	*mg./100 cc.*
Calcium		10.1		10.1
Magnesium	4.77	3.64	6.05	2.42
Chloride	280.0		270.0	
Inorganic phosphorus	19.3	14.9	11.01	8.73
Acid soluble phosphorus	26.0	15.2	51.0	9.7
Lipid phosphorus		3.3		4.8
Amino acid nitrogen	9.58	7.36	9.34	7.1

not show much change in the phosphorous partition. This is probably due to metabolism of fats from fat reserve. In the case of pig No. 6117 there was very little tissue fat. No significant impairment in nitrogen metabolism was indicated by the normal amino acid content of the blood of both pigs.

The relatively rapid and widespread growth of the adenoma at this early age and the fact that papillomatous growths of virus etiology are sometimes encountered on the tongue and in the oral cavity suggested the possible presence of an infectious agent. The affected intestinal tissue and tumors of pig No. 6117 were rinsed with distilled water and one portion was preserved in 50 per cent sterile glycerin overnight, while the remainder was placed in saline in a refrigerator. Ten cc. of a Berkefeld N filtrate was injected intraperitoneally in a 5 months old pig. Material from the same filtrate was placed on the tongue by means of a hypodermic syringe and needle. At the same time the mucous membrane of the tongue was scarified. Additional filtrate was injected into

the submucosa of the tongue and into the cutis and subcutis of the chest region. About 250 cc. of filtrate was given by stomach tube to a 2nd pig. A 3rd pig of the same age was fed on 2 successive days finely cut adenomatous tissue that had been stored in 50 per cent glycerin overnight. A 4th pig was fed finely cut tissue from the adenomatous intestine which had been collected and held in saline solution. When the 4 pigs were subsequently destroyed no evidence of adenomatous growth could be demonstrated.

Bacteriological examination of the tissues of pig No. 6117 failed to demonstrate the presence of *S. suipestifer*. The failure of the pigs to develop enteritis when fed unfiltered intestinal tissue from pig No. 6117 constitutes further evidence of the absence of an infectious form of enteritis.

Summary

A case of rapidly developing intestinal adenomatosis in the pig is described. The process did not appear to be associated with an infectious agent. The low grade invasive power of *B. coli* in the pig and its absence in the lesions of the small intestine indicate a lack of correlation with the newgrowth.

REFERENCE

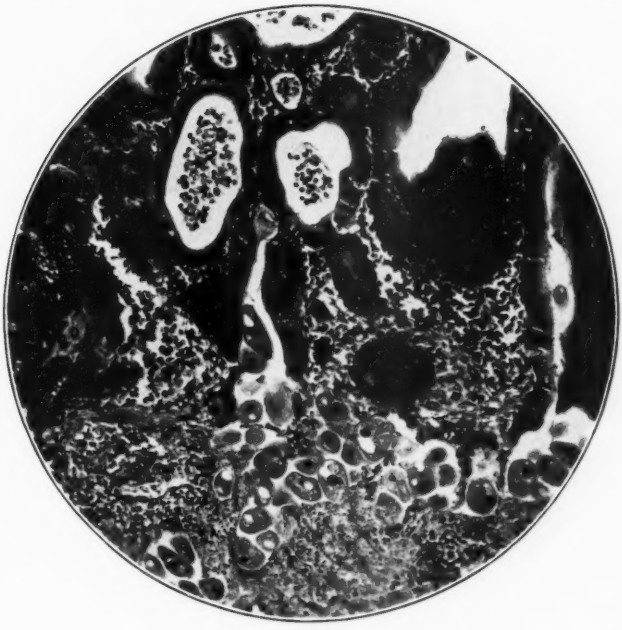
1. Biester, H. E., and Schwarte, L. H. Intestinal adenoma in swine. *Am. J. Path.*, 1931, 7, 175–185.

DESCRIPTION OF PLATE

PLATE 66

FIG. 1. Section of adenomatous tissue from the cecum with croupous membrane containing *B. coli*.

FIG. 2. Section from the ileum showing the adenomatous growth with croupous membrane formation. Red blood cells are present in the depths of the exudate. No *B. coli* are present.



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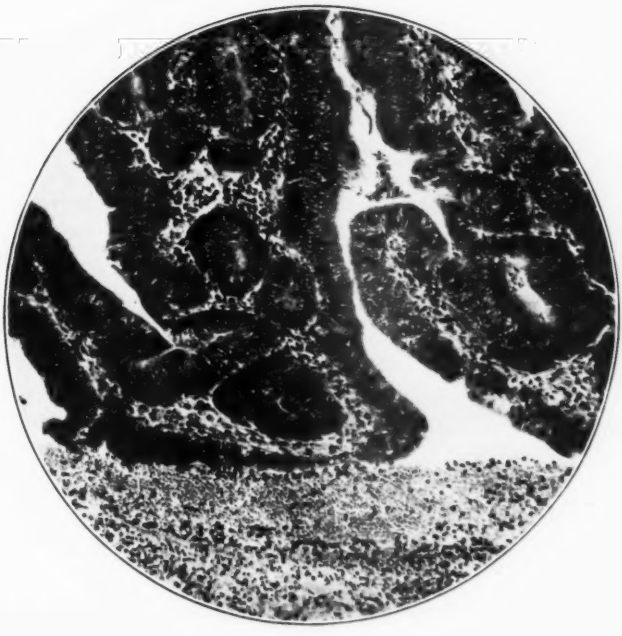

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Biester, Schwarte and Eveleth Intestinal Adenoma in a Pig